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THE EFFECT OF ALGAE ON BOD MEASUREMENTS

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THE EFFECT OF ALGAE ON BOD MEASUREMENTS

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THE EFFECT OF ALGAE ON BOD MEASUREMENTS

I. The Factors Affecting the Measurement of Algal Biochemical Oxygen Demand (BOD)

George F. Fitzgerald

Introduction

The aim of this project was to measure the BOD of various species of algae alone and in association with bacteria under incubation conditions of 5 days' time in the dark and at a temperature of 20°C to determine if algae have an effect on BOD measurements under the standard conditions.

Initial studies have been carried out using the green alga, Chlorella pyrenoidosa (Wis. 2005). This alga is especially adaptable to various culture conditions and can be used as a basis for comparison with other algae having more restricted culture conditions.

The culture procedures used to supply Chlorella for the BOD tests are described and the growth rates of this alga in four different media were measured. Because there is little information in the literature on the measurement of dissolved oxygen (DO) in the presence of algae, tests were carried out to observe if algae affected this measurement. Another series of tests were made to determine if the algae used in BOD tests under the standard conditions would survive the 5 days of darkness in the presence of bacteria. The effect of adding sewage bacteria to cultures of algae was also determined, both on the activity of the algae and the effect of the culture supernatant in the BOD test.

As soon as some of the preliminary studies on the effect of algae on the various measurements involved and the effects of mixing algae and bacteria were known, studies were made of the factors affecting the activity of the algae during the BOD tests. The various factors studied have been the amount of algae, the source of algae (i.e., the medium in which the algae were cultured), and the age of the algal cultures at the time of use in the BOD test, (i.e., the factors causing senility in algal cultures using two media, Gorham's and secondary sewage effluent). Other algae, representing both green and blue-green species, have also been studied in comparative tests with Chlorella (Section II).

Studies on the influence of the dilution medium in the BOD test have been made. Comparison of the BOD of Chlorella in dilution water, algal culture media, and in lake waters, as well as seasonal changes in the influence of lake waters are reported (Section III).

Materials and Methods

The Chlorella pyrenoidosa (Wis. 2005) used was maintained as a pure culture and a volume of about 15 ml was used to inoculate each experimental culture. A 2-liter Erlenmeyer flask with 1500 ml of medium was used as a culture vessel. A cotton plug supported a 6-mm diameter glass tube with a constricted end that was used for bubbling a 1.25 per cent CO_2-air mixture through the culture. Each bubbler was attached by surgical rubber tubing to a 25 x 200 ml glass tube containing cotton as a filter. A 50-mm teflon-covered magnet was placed in each flask. The culture vessel, media, magnet, and filter were sterilized by autoclaving. Inoculations were made with sterile syringes. Motors (1/100 HP shaded pole motor, Dayton Elec. Mfg. Co., Chicago, Ill.) with magnets attached to their shafts were mounted vertically on wooden frames below the culture vessels and the speed of 15 magnetically stirred cultures was controlled with one variable transformer. The algae were cultured under continuous light (180 ft candles) in a constant temperature ($23 \pm 1^{\circ}$ C) culture room.

The composition of the four media used for culturing Chlorella are listed in Table I-1. The dilution water medium is BOD dilution water (1) plus 30 mg/l of nitrogen from $NaNO_3$. The pH of this medium is near 7.4 at harvest time. Allen's medium (2) is also a neutral medium, the final pH being near 7.0. Myers' medium (3) is more acidic, the pH being near 6.5. Gorham's medium (4) is alkaline, with a final pH of near 9.0.

In addition to the above synthetic media, experiments were also carried out with Chlorella grown in autoclaved secondary effluent from the Madison Sewage Treatment Plant. The general composition of this effluent was approximately 20 mg NH_3-N/l, 7 mg PO_4-P/l, 950 mg total solids/l, 380 mg methyl orange alkalinity (as $CaCO_3$)/l, 10-30 mg BOD/l, and a pH of 7.8.

The algae to be used in an experiment were centrifuged from their culture solution after their concentration was measured, the supernatant was discarded, the cells were washed with pH 7 PO_4-buffer, and recentrifuged. The cells were then suspended in a convenient volume of PO_4-buffer and measured samples added to seeded dilution water for mixing and filling of BOD bottles. Duplicate bottles were used to determine the initial DO concentrations and triplicate bottles were used for analysis after 5 days' incubation at 20°C in a dark room. Bottles containing algae were covered with black hoods as a further precaution to prevent light from entering during the incubation period. Measurements were made as suggested in Standard Methods (1).

The procedure used to measure the amount of oxygen absorbed by algae during the 5-day BOD determinations was to measure the oxygen used

Table I-1

Media Used for Culture of Chlorella for BOD Experiments

A. Concentration of salts used in media (mg/l)

Salt	Dilution Water	Gorham's	Allen's	Myers'
NH_4Cl	1.7		50	
$NaNO_3$	182.0	496	1000	
KNO_3				1210
KH_2PO_4	8.5			1230
K_2HPO_4	21.75	39	250	
$NaHPO_4 \cdot 7H_2O$	33.4			
Fe Citrate		6		
$FeCl_3$	0.15		3	
$Fe_2(SO_4)_3$				52
$MgSO_4 \cdot 7H_2O$	22.5	75	513	2460
$CaCl_2$	27.5	27	50	
$Na_2SiO_3 \cdot 9H_2O$		58		
Citric acid		6		
EDTA		1		
Na Citrate				195
Na_2CO_3		20		
Total Salts	297.5	728	1866	5147

B. Concentration of essential ions in media (mg/l)

ion	Dilution Water	Gorham's	Allen's	Myers'
N	30.0	82	178	168
P	10.0	7	45	295
Fe	0.05	1	1	15
Mg	2.0	7	50	243
Ca	10.0	10	18	
S	3.0	10	67	323
K	12.0	9	56	353

by seeded dilution water (24-hour settled sewage seed) and seeded dilution water plus algae. The difference in the oxygen depletion was referred to as being caused by the algae and reported as $\triangle$ DO per mg algae.

Results

1. Growth of Chlorella in Different Media

Because there is no one growth measurement common to all algae laboratories, growth measurements were made on cultures as suspended solids, volatile solids, COD (dichromate method with Ag_2SO_4 catalyst (1)), cell counts, and packed volume. The general relationships between these measurements of Chlorella cultures of approximately 10 days of age are presented in Table I-2.

2. Effect of Algae on Dissolved Oxygen Measurement

The effect of the presence of algae in measuring dissolved oxygen (DO) by the Alsterberg (azide) modification of the Winkler method (Standard Methods (1)) was determined.

It was found that concentrations of Chlorella as high as 4×10^6 cells/ml did not appreciably affect the measurement of DO. The end point was slightly obscured, but experience with the measurement overcame this disadvantage. Once the manganous sulfate and alkaline iodide reagents were added, it was found that up to 2 hour intervals between the addition of the sulfuric acid or titration did not affect the DO measured in the presence of Chlorella (5×10^6 cells/ml).

3. Effect of Seed Volume on Activity of Algae

It is well known that considerable variation may be expected in the activity of different settled sewage seeds. An attempt was made to always obtain samples of raw sewage at the same time of day for each experiment, but there was a variation in the ratio of seed activity per volume depending upon the day of the week. Therefore, four experiments were carried out in which the oxygen absorbed by different volumes (3.0 to 30 ml/l) of 20-24 hour settled sewage seed in dilution water was measured as well as the total amount of oxygen absorbed when a definite amount of Chlorella from a dilution water culture was added to each seed volume in dilution water. These data are presented in Figure I-1.

For example in Experiment 11, data for line A show the variation in oxygen absorbed for variations in seed volume. Data for line A' which represents seed plus Chlorella cells show increased oxygen absorption, and

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Table I-2

Relative Growth of Chlorella after 10 Days in Different Media

Culture Medium	Susp. S. (mg/l)	Vol. S. (mg/l)	COD (mg/l)	Cell Count (cell/ml x 10^6)	Pack Vol. (ml/l)
Dilution Water	125	100	175	12.5	0.6
Gorham's	175	200	250	5.0	0.8
Allen's	375	350	550	50.0	2.0
Myers'	375	350	550	50.0	2.0

the increment of increase is constant regardless of seed concentration. This relationship was observed from the data obtained in the other experiments with varying amounts of algae as described in Figure I-1.

It is evident from these data that a variation of 10 fold in seed volume or 7 fold in oxygen absorption due to the seed had little or no effect on the amount of oxygen absorbed by the added amount of algae.

4. Effect of Contaminating Chlorella Cultures with Sewage on Subsequent BOD Measurements

Settled sewage was added to cultures of Chlorella (1 to 5 ml sewage per liter of culture) that had previously been sampled for BOD measurements. After about 5 additional days of incubation in the culture room under normal growth conditions the cultures were again sampled for BOD measurements. The effects of both washed algal cells and of the culture supernatants were measured in seeded dilution water tests.

A total of 45 tests were carried out on algae, from dilution water, Allen's, and Myers' media, that had been incubated with sewage. It was found that the average activity of the algae from contaminated cultures was within the experimental limits for that medium as compared with algae from pure cultures. From these data it was concluded that algae from either pure or contaminated cultures would have essentially the same effect on the BOD measurement.

There was a different effect of contaminating Chlorella cultures with sewage on the results in BOD measurements produced by the supernatants from the cultures. It was found in 12 tests with cultures of Chlorella from dilution water, Allen's, and Myers' media that the effect of contamination with the sewage caused a decrease in the BOD of the supernatant to the extent that the BOD was 1/5 or less after incubation with sewage for 5 days as compared with the supernatant of the cultures prior to contamination. This is to be expected, of course, because the organic matter accumulated in algal culture supernatants had been found to increase the activity of sewage seed and, thus, was further proof that some of this material could be broken down by sewage organisms.

5. Comparison of Live and Dead Algae

It has been pointed out in the literature (5, 10, 11) that dead algae had considerably higher oxygen demands than live algae. In order to check this, a series of BOD tests were made with live algae from Allen's medium, similar cells which had been autoclaved, and cells which had been freeze-dried.

Experiments were first carried out to test the viability of Chlorella cells which had (1) no treatment, (2) had been frozen only, and (3) which had been freeze-dried (3.6% final moisture content as compared to 83% moisture in fresh cells). Five tests (total of 11 samples for each treatment) indicated that cells suspended in either distilled water or autoclaved 1 week old culture supernatant, and then frozen for the same period of time required in the freeze-drying process (1 to 3 days), had a viability of about 20% as compared to unfrozen controls. No cells were viable in freeze-dried samples of up to 10,000 cells, but in one experiment, 4 algae cells out of 500,000 cells survived the freeze-drying treatment. Therefore, it may be concluded that freeze-dried Chlorella cells for these tests are, by and large, non-viable, but may still be considered to be chemically intact much the same as live cells as compared to cells which have been killed by autoclaving.

The results of 8 BOD tests each with fresh, freeze-dried, and autoclaved Chlorella cells indicated that the average activity factors (Δ DO/mg S.S.) were 0.127, 0.439, and 0.481, respectively. The ratio of activity of fresh cells to freeze-dried cells was 3.46 when comparing Δ DO/mg S.S., 5.93 for Δ DO/10^9 cells, and 4.03 for Δ DO/ml packed cells. The ratio of activity of fresh cells to autoclaved cells was 3.79 for Δ DO/ml S.S., 3.11 for Δ DO/10^9 cells, and 4.86 for Δ DO/ml packed cells. Thus, it has been confirmed that dead algae cells exert higher BOD's than live cells, and the BOD of cells killed by freeze-drying are nearly as great as the BOD of cells killed by autoclaving.

6. Comparison of Viability and Photosynthetic Capacity

Previous tests had indicated that dead cells exerted a higher BOD than live cells and other tests showed that algae from the dilution water medium also exerted a higher BOD than algae from Allen's medium. It was thought that perhaps the reason for the higher BOD activity factors for algae from the dilution water medium as compared to Allen's medium was the result of the presence of more dead cells in the BOD test of algae from the dilution water medium, thus stimulating the respiration of the seed bacteria and giving apparent higher algal activity factors. If this were true, one could expect a greater decrease in viable cells during the BOD test with algae from dilution water medium as compared with algae from Allen's medium, and a greater decrease in the amount of oxygen these algae could produce when exposed to light.

Viability tests of the mixture of algae and seeded BOD dilution water before and after incubation were made by dilution samples of the mixture using sterile, pH 7.2 phosphate buffer (as described in Standard Methods (1)) and plating in petri dishes with tryptone glucose extract agar. Some samples

required homogenizing in a Waring blender to break up clumps of algae before dilution and plating. The viable algae cells forming colonies under these conditions were counted after incubation in the constant light culture room for at least 10 days. It might be pointed out that the algal cell counts in some plates were less than in duplicate plates, or algal colonies were smaller in some duplicate plates, inhibition resulting because of the presence of certain types of bacteria.

Comparisons of cell counts and viability measurements in algae-seeded mixtures at the time of mixing (zero time) and after 5 days' incubation indicated that for algae from Allen's medium, 85% of the cells counted at zero time (31 tests) and 89% of cells counted after 5 days (25 tests) were found to be viable cells. Algae from dilution water cultures had a survival, as compared to cell counts, of 72% at zero time (35 tests) and 70% after 5 days (32 tests).

The results of viability tests comparing the number of cells viable at the time BOD tests were started (zero time) with the number of viable cells found in BOD bottles after 5 days' incubation in the dark indicated that 88% of the cells from Allen's medium (17 tests) and 76% of cells from dilution water medium (34 tests) were alive after the incubation period. However, in comparing data of individual experiments it was found that the amount of oxygen used per mg of suspended solids (Δ DO/mg S.S.) was not necessarily related to the results of viability tests; essentially the same Δ DO/mg S.S. was obtained from experiments which had 100% recovery of cells as from experiments in which there was only a 52% recovery (dilution water algae).

The photosynthetic capacity of the algae-seed mixtures was measured at zero time and after 5 days' incubation by comparing duplicate DO analyses of samples held in the dark with samples exposed to 350 ft. candles of light for 2 hours just before analysis. The amount of oxygen that the algae produced in the light in excess of that used in the dark was controlled by the physical conditions of the medium at the time of analysis, such as CO_2 content of the water in the BOD bottles, as well as the physiological state of the algae. However, since this test was used to compare algae from different sources, but in the same medium after the same incubation times, it was considered to be a reliable comparative test for the activity of the different algae at that particular time.

The amount or rate of oxygen produced was expressed as the change in oxygen per mg suspended solids for the 2-hour period. The average rate of 35 tests of algae from Allen's medium measured at the start of the BOD tests was 0.093 Δ DO/mg S.S., while it was 0.083 Δ DO/mg S.S. when tested after 5 days' incubation, a decrease of 11%. It should be pointed out that the average of these two rates of oxygen production, 0.088 Δ DO/mg S.S., for a

two-hour period of light exposure is equivalent to 5.28 mg of oxygen production/l for a 5-day period, or a considerably higher rate than the average respiratory rate for algae from this source (0.105 Δ DO/mg S.S.)(see also reference 10).

The photosynthetic capacity of algae from dilution water medium (32 tests) had an average rate of 0.056 Δ DO/mg S.S. at zero time and 0.024 Δ DO/mg S.S. after 5 days' incubation, a decrease, in 5 days' time, in photosynthetic capacity of 57%.

In comparing the photosynthetic capacity of algae from Allen's medium with algae from dilution water medium (algae from Allen's medium were 40% more active at zero time and 71% more active after the 5 days of incubation than algae from dilution water medium) it must be remembered that the average amount of oxygen used in the BOD test for algae from Allen's medium was 0.105 Δ DO/mg S.S. (52 tests), while it was 0.190 Δ DO/mg S.S. for algae from dilution water medium (87 tests), or the apparent respiratory rate of algae from dilution water medium measured in seeded dilution water was 45% higher than algae from Allen's medium. Therefore, it would seem that the higher activity in the BOD test of algae from dilution water medium may be due to the death of some cells from dilution water medium and resulting stimulation of bacterial respiration.

7. Comparison of Algal Activity Rates Under Bacterial-Free Conditions

Previous data had indicated that, although the apparent respiratory rate of algae from the dilution water medium was higher than that of algae from Allen's medium, the photosynthetic capacity of algae from dilution water medium was less. As was pointed out, this apparent higher respiratory activity may be due to more stimulation of the bacteria present in the case of algae from dilution water cultures than in the case of algae from Allen's medium. Therefore, BOD tests were made under bacterial-free conditions.

The procedure used to measure the amount of oxygen that algae would use in 5 days, in the absence of bacteria, was to add algae from pure cultures to sterile BOD dilution water and siphon the mixture to sterile BOD bottles. Duplicate flasks were analysed for DO at the beginning and after 5 days' incubation in the same manner as in normal BOD tests. At the same time as these tests were run, duplicate tests were also run in seeded dilution water using identical samples of algae.

The results of the bacterial-free and seeded BOD tests indicated that 12% more oxygen was consumed per mg suspended solids in seeded BOD tests than in bacterial-free BOD tests with algae from Allen's medium (8 tests), and 34% more with algae from the dilution water medium (12 tests). The

average BOD of algae from Allen's medium (bacterial-free) was 0.120 as compared to 0.105 /. DO/mg S.S. for the average for all seeded BOD tests with algae from Allen's medium. However, the average BOD of dilution water algae in the bacterial-free tests was 0.127 as compared to 0.190 /. DO/mg S.S. for all seeded tests. The similarity of the activity of these algae under bacterial-free conditions (Allen's: 0.120, and dilution water: 0.127, . DO/mg S.S.) should be noted.

Comparisons of measurements of the photosynthetic capacity of the algae from the two media under seeded and bacterial-free conditions indicated that there was little difference in the photosynthetic activity of algae from Allen's medium at zero time (0.093 seeded vs. 0.095 sterile △ DO/mg S.S.). However, in the case of algae from the dilution water medium, the average zero time photosynthetic capacity increased from 0.056 △ DO/mg S.S. for the seeded tests to 0.071 /. DO/mg S.S. for bacterial-free tests. The average photosynthetic capacity of all seeded tests after 5 days' incubation for dilution water algae (32 tests) was 0.024 △ DO/mg S.S. and for the 12 seeded tests, run in parallel with the bacterial-free tests, was 0.027 △ DO/mg S.S. In contrast, the photosynthetic capacity of algae from this medium under conditions of sterile incubation for 5 days was 0.054 △ DO/mg S.S., or exactly twice the rate of tests run in seeded BOD bottles.

8. Daily and Long Term BOD Studies

A series of BOD tests have been run to establish the rate of oxygen used by algae on a daily basis and for long periods of time, up to 16 days. The tests were set up as for normal BOD measurements except that duplicate samples were analyzed at the times reported.

Figure I-2 presents data from an experiment showing the daily change in dissolved oxygen in BOD bottles of seeded dilution water, seed plus 13.4 mg/l, and seed plus 26.8 mg/l algae from Allen's medium. It is evident from these data that oxygen utilization by the algae is fairly uniform over the 5-day period in the dark.

Figure I-3 presents data obtained when algae from Allen's medium was added to seeded dilution water (13.4 mg algae/l) and some bottles were either kept in the dark until analyzed (in duplicate), exposed to 2 hours of 350 ft. candles of light and then kept in the dark, or exposed to 2 hours of 350 ft. candles of light just before analysis after incubation in the dark. It is evident from these data that the initial DO concentrations of samples can be raised by exposure of the samples with algae to light with little significant change in the rate of oxygen utilization from samples not exposed to an initial light treatment (there was an average of 5% increase in activity of samples exposed to light as compared to samples without light treatment - 6 samples).

The data showing the level to which the DO concentrations are raised in samples exposed to light just before analysis on a daily basis (Figure I-3) indicate that for the 5-day period with algae from Allen's medium there is little change in the photosynthetic capacity of these algae (average oxygen production for 35 samples at zero time was 0.093 $\triangle$ DO/mg S.S. and 0.083/$\triangle$ DO/mg S.S. for 5-day samples).

BOD tests were also run for as long as 16 days to determine the rate at which oxygen was consumed by algae over periods of time in excess of the usual 5 days' time used in BOD tests. Figure I-4 shows the rate at which oxygen was consumed by sewage seed (at 6 times the amount used to seed the dilution water used with the algae tests) and the rate of oxygen utilization by algae from Allen's medium (14.2 mg/l) as compared with that of algae from dilution water medium (16.5 mg/l).

Figure I-5 presents the rate at which oxygen was used by 28 mg/l of algae from Allen's medium over a 16-day period, as well as the amount of oxygen produced by exposure of samples to 350 ft candles of light for 2 hours prior to analysis. It can be seen that the BOD bottles became anaerobic (DO exhausted) at about 9 days' time. However, the algae present continued to produce oxygen when exposed to light, but at a decreasing rate. Viability tests of samples from these bottles at zero, 5, 12, 14, and 16 days indicated no loss in viability (average per cent viable cells were 100, 94, 120, 123, and 115, respectively). Thus, these data confirm the results of Golueke, et al. (6) who found Chlorella and Scenedesmus to be relatively resistant to anaerobic digestion and of Finkle, et al. (7) who found Chlorella to be viable after 70 days in the dark.

9. Effect of Glucose and Fructose on BOD of Chlorella

The data reported thus far have dealt with the BOD of Chlorella when measured in an inorganic medium with the addition of minor amounts of dissolved or suspended organic matter in the form of settled sewage (the BOD of seeded dilution water being about 0.5 mg/l). In order to demonstrate the complexity of the relationship between the BOD of algae in such an environment to the BOD of algae in an environment containing organic materials in appreciable quantities, a series of 5 tests were carried out using glucose and fructose. These 2 organic compounds represent readily available compounds to bacteria, while being selectively available to Chlorella: glucose being known to be an available source of energy (8, 9), whereas fructose has been shown to be relatively unavailable to certain species of algae, including those used in these tests (9).

These tests were carried out in the normal manner of testing seeded dilution water with or without algae and seeded dilution water containing 4.3 mg of glucose or fructose per liter with or without algae. The results of the five

tests with fructose as an added substrate have indicated that the presence of Chlorella had no effect on the bacterial BOD of fructose (less than 10% variation with controls). Also, stimulation of bacterial respiration by the presence of fructose had no effect on the BOD of Chlorella (less than 5% variation). In effect, the BOD of Chlorella in the presence of fructose was merely additive to the BOD of fructose. In contrast, in the studies in which glucose was used as an added substrate, the algae had a decided effect on the bacterial BOD. The presence of Chlorella decreased the bacterial BOD of glucose by about 50%. This effect could be interpreted to indicate that the algae absorbed some of the glucose from solution, but the effect of this absorbed glucose on the apparent BOD of the algae was not as great as an equivalent amount of glucose absorbed by sewage bacteria.

Summary and Conclusions

1. The rate of growth of Chlorella in different media has been followed by measurements in the culture of suspended solids, volatile solids, COD, cell counts, and packed volume.

2.It was found the DO could be readily determined using the Winkler method in solutions containing Chlorella in concentrations at least as high as 5×10^6 cells per ml.

3. The activity of Chlorella in seeded dilution water in the BOD test appears to be independent of the volume of seed added (in the range of 3 to 30 ml seed/l).

4. There appeared to be no difference in the activity of Chlorella in the BOD test whether the original culture was bacterial-free or contaminated with sewage bacteria (1 to 5 ml sewage/l). However, the addition of sewage to cultures caused a 5-fold loss in the activity due to the supernatant of the algal cultures when tested in BOD tests.

5. The BOD of autoclaved or freeze-dried Chlorella cells were approximately 4 fold that of living cells.

6. No consistent relationship could be obtained between the amount of oxygen used by Chlorella in the BOD test and the number of viable cells measured by dilution and plating of samples after 5 days' incubation.

7. The photosynthetic capacity of the algae before and after incubation in the BOD test indicated the physiological state of the algae. Tests showed that this measurement could be used to indicate if algae were still active after different periods of dark incubation and the relative activity of algae from different cultures.

8. Tests of the BOD of Chlorella under seeded and bacterial-free conditions indicated the effect of algae from Allen's medium were merely additive to the respiration of bacteria, whereas algae from the more dilute dilution water medium might have caused a slight stimulation to the respiration of bacteria present.

9. Daily and long-term studies of Chlorella in the BOD test showed that the rate of oxygen utilization was quite uniform until the BOD bottles became anaerobic. It was found that Chlorella could continue to produce oxygen by photosynthesis for at least 7 days after the BOD bottles became anaerobic.

10. The rate of oxygen utilization by Chlorella in the BOD test was found to be independent of any increase in bacterial respiration due to the presence of fructose. However, in the presence of glucose in the BOD test, the algae had a decided effect on the bacterial BOD of this organic compound.

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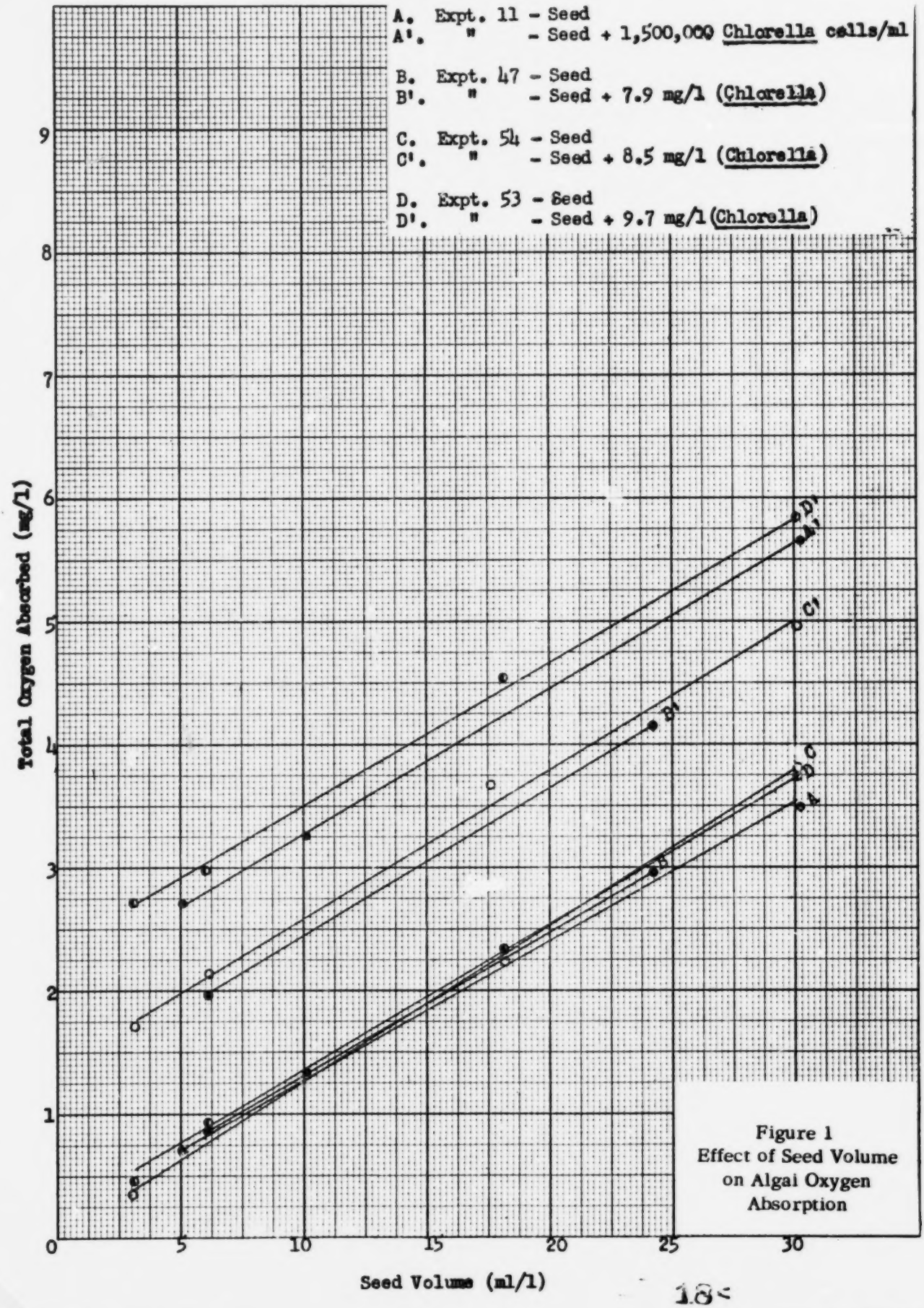

Figure 1
Effect of Seed Volume
on Algal Oxygen
Absorption

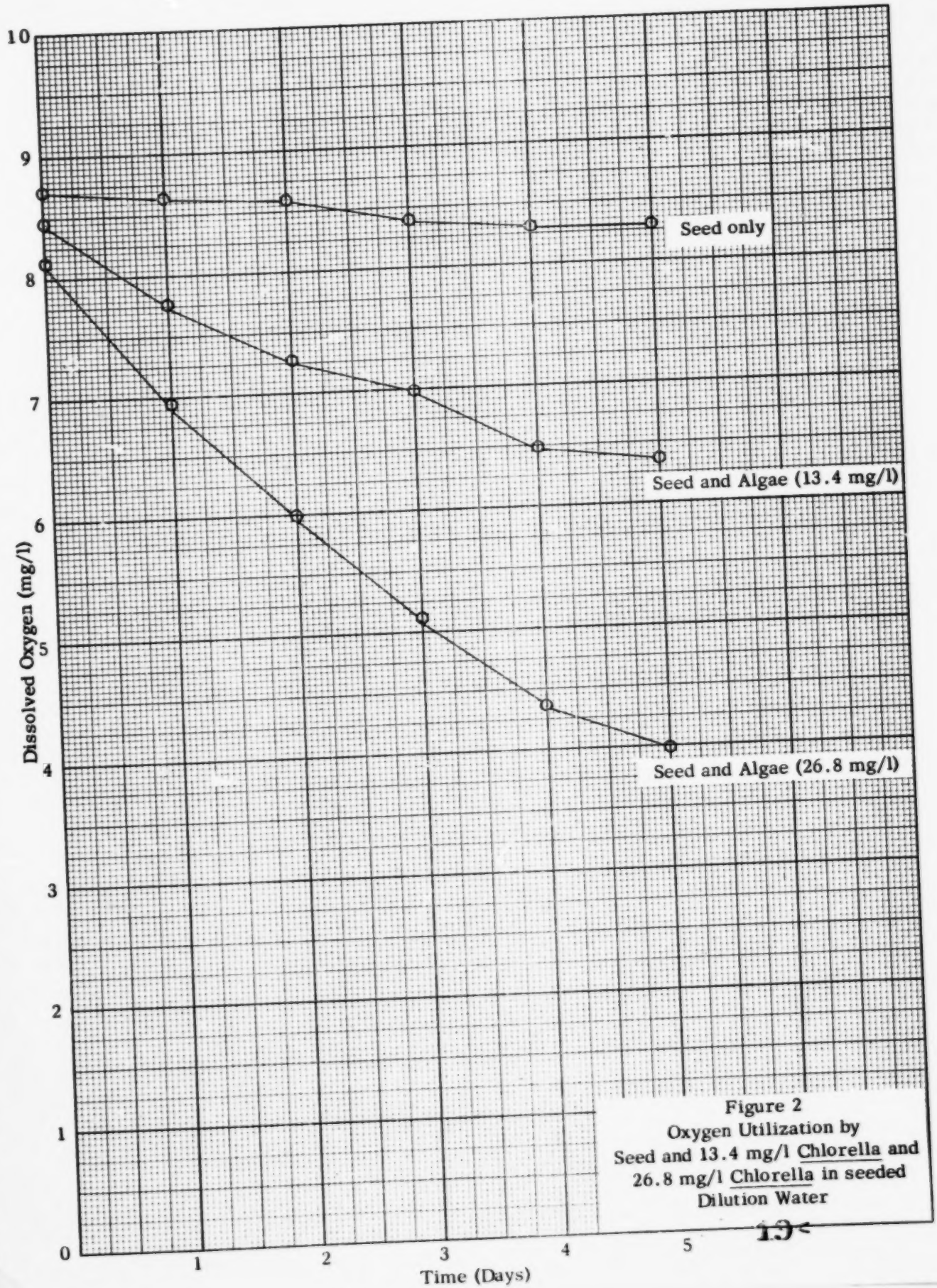

Figure 2
Oxygen Utilization by
Seed and 13.4 mg/l Chlorella and
26.8 mg/l Chlorella in seeded
Dilution Water

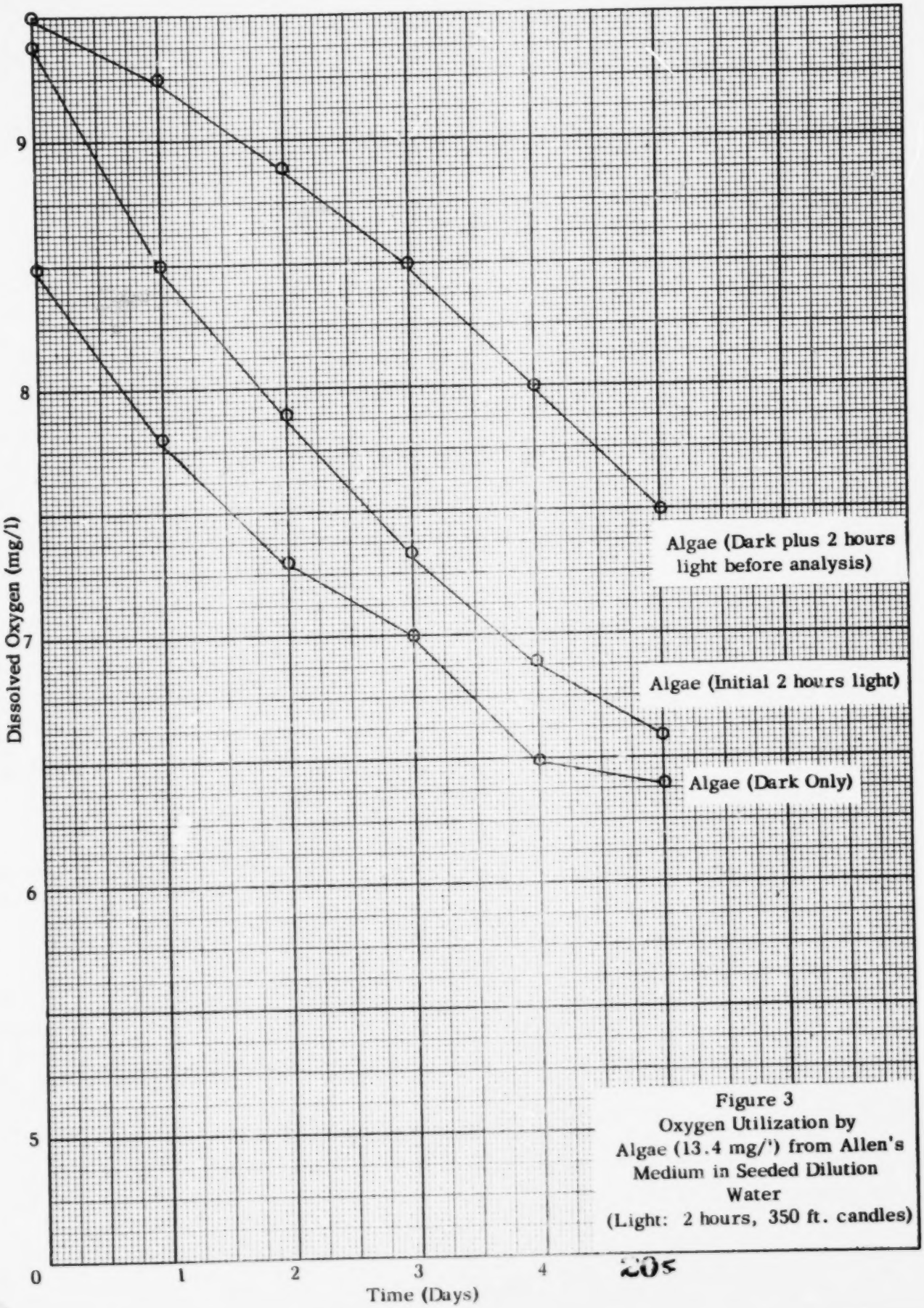

Figure 3
Oxygen Utilization by Algae (13.4 mg/l) from Allen's Medium in Seeded Dilution Water
(Light: 2 hours, 350 ft. candles)

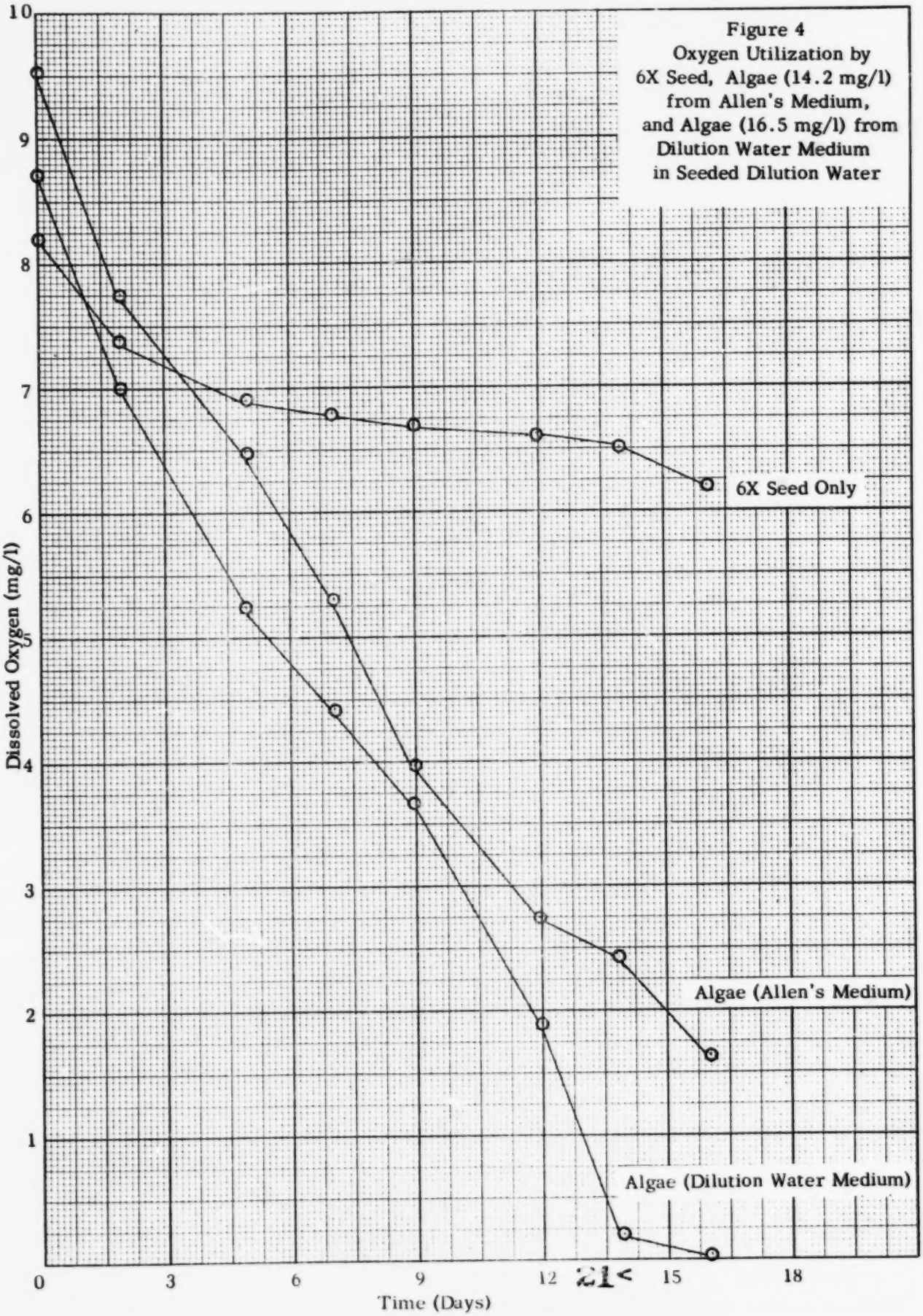
Figure 4
Oxygen Utilization by
6X Seed, Algae (14.2 mg/l)
from Allen's Medium,
and Algae (16.5 mg/l) from
Dilution Water Medium
in Seeded Dilution Water
6X Seed Only
Algae (Allen's Medium)
Algae (Dilution Water Medium)
Dissolved Oxygen (mg/l)
Time (Days)
0
3
6
9
12
15
18
1
2
3
4
5
6
7
8
9
10

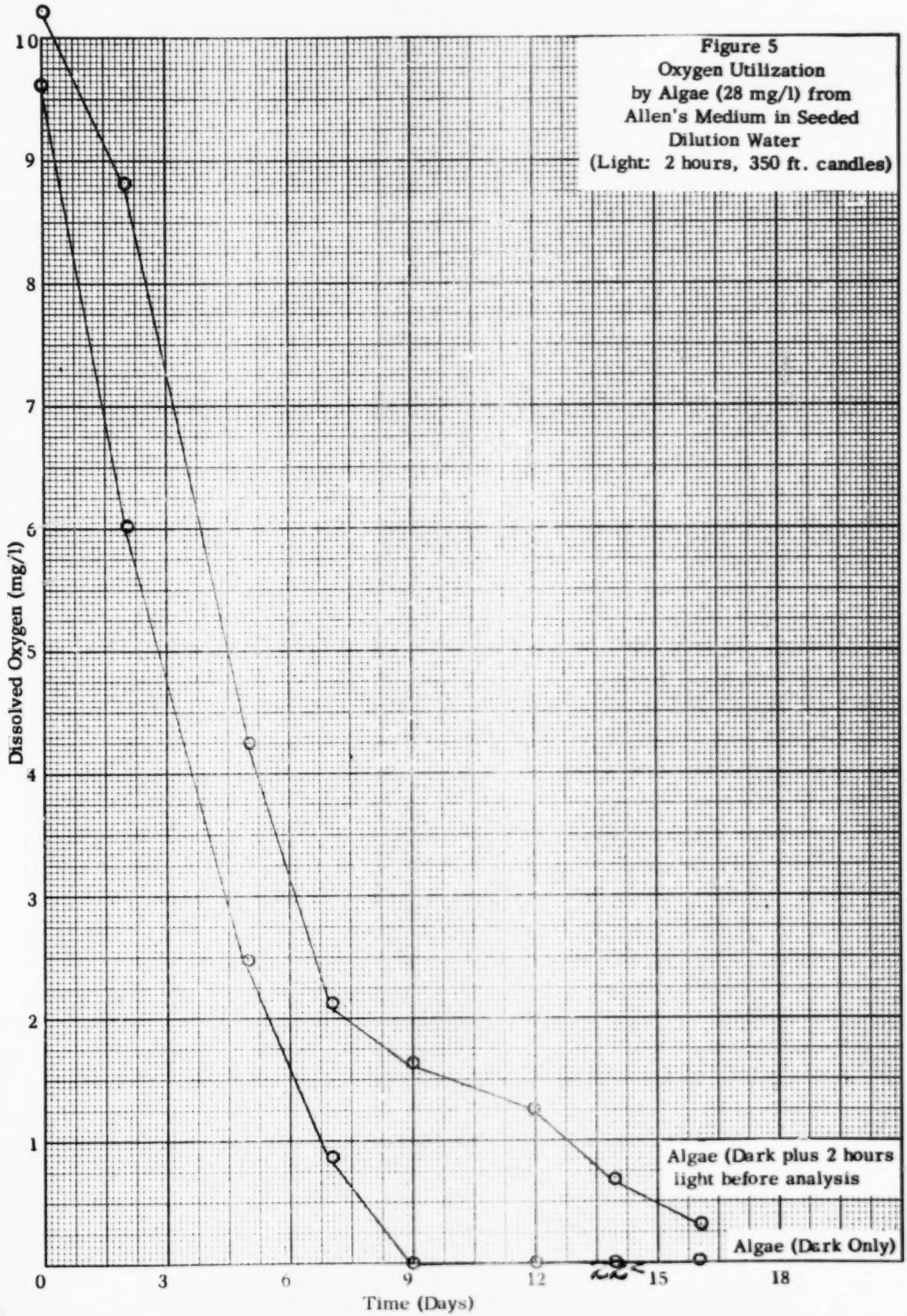
Figure 5
Oxygen Utilization
by Algae (28 mg/l) from
Allen's Medium in Seeded
Dilution Water
(Light: 2 hours, 350 ft. candles)
Dissolved Oxygen (mg/l)
Time (Days)
Algae (Dark plus 2 hours
light before analysis
Algae (Dark Only)
0
3
6
9
12
15
18
1
2
3
4
5
6
7
8
9
10

THE EFFECT OF ALGAE ON BOD MEASUREMENTS

II. The Effect of the Culture Medium and Species of Algae

George P. Fitzgerald

Introduction

Early experiments using 5 different culture media had indicated that there was a wide range in the activity of Chlorella in the BOD test, depending upon the medium this alga had been cultured in prior to the BOD test. Studies were made of the influence the culture medium may have on the activity of Chlorella and what factors may affect this activity.

As an extension of the data collected using Chlorella as a test organism, further studies were carried out comparing the activity of the blue-green bloom forming alga, Microcystis aeruginosa (Wis. 1036) to that of Chlorella. Also, the activity of other green and blue-green algae was measured: Scenedesmus dimorpha, Nostoc muscorum, and Gloeotrichia echinulata.

Results

1. The Effect of the Culture Medium

Preliminary experiments indicated that the activity of Chlorella in the BOD test could range from 0.06 to 0.31 mg O_2 used per mg suspended solids per l (Δ DO/mg S.S.), depending upon the medium in which the algae had been cultured. The distressing observation from these data was that the apparent activity of the algae from the different media appeared to be inversely correlated to the potential growth rate of Chlorella in the media, that is, algae from the two more concentrated media, Myers' and Allen's, showed less activity in the BOD test than algae from the very dilute dilution water medium. Other data, such as the results of bacterial-free BOD tests, however, indicated that, perhaps, the measured BOD of algae from the dilution water medium was really a combination of algal respiration and stimulated bacterial respiration.

Because cell counts and viability tests by the dilution technique gave little correlation to other data, measurements of the photosynthetic capacity of algae from Allen's and dilution water media at the start and end of BOD tests were made. These data indicated that the photosynthetic capacity of algae from Allen's medium decreased 11% whereas there was a decrease of 57% with algae from the dilution water medium over a period of 5 days' time.

A re-evaluation of the effect of the different culture media on the activity of Chlorella in the BOD test was carried out by comparing the apparent respiration of the algae over the 5-day period with the photosynthetic capacity of the algae as measured after 5 days' incubation in the dark. The results are presented in Table II-1.

These data indicate that algae grown in the three media, Myers', Allen's, and Gorham's have about the same photosynthetic capacity after 5 days' incubation in the dark, but there is about a 50% increase in the activity of the algae in the BOD test. The algae from the dilution water medium have the highest activity in the BOD test, but demonstrate the lowest activity in the photosynthetic capacity test.

2. The Effect of the Age of the Culture on Algal Activity

In order to measure the effect the age of a culture had on the activity of algae in the BOD test, algae were used from cultures considered young or old, depending upon the growth rate of the algae in the particular medium. A comparison was made of these two "ages" of cultures for dilution water, Allen's, Myers' and Gorham's (air for aeration) media. The results of these tests are summarized in Figure II-1 as the amount of oxygen used by the algae per mg of suspended solids ($\triangle$ DO/mg S.S.) for the different ages of the cultures. The solid circles indicate the average activity factor for each age of the culture media tested and the heavy lines running through the circles indicate the range of ages used. Light lines are used to make the lines for each medium continuous. The number of samples used to determine the average value is indicated by the figure in parentheses at each dot. The ranges of pH of the media are also presented. The pH values presented are for the cultures at the time of harvesting. There was little change in the pH of the cultures throughout the growth period.

It is evident that the average algal activity factor for young and old cultures of dilution water (0.181 and 0.184 $\triangle$ DO/mg S.S.) and Allen's medium (0.106 and 0.116 $\triangle$ DO/mg S.S.) indicate comparitively little change in activity per mg of cell material as the age of the culture increases within the limits tested. However, in the case of algae cultured in the other two media there was a definite loss in activity in older cultures as compared with young cultures: the average value for young and old cultures was 0.094 and 0.063 $\triangle$ DO/mg S.S. for algae from Myers' medium and 0.163 and 0.124 $\triangle$ DO/mg S.S. for algae from Gorham's medium.

Inasmuch as the two media (dilution water and Allen's) that had the least decrease in activity with age of the culture were approximately neutral in pH value, it might be possible that pH values further removed from the neutral value may be the principal cause of the senility of the cultures.

Table II-1

The Effect of the Culture Medium on the Activity of Chlorella in the BOD Test

Culture Medium	pH	No. of Tests	BOD* Range	BOD* Ave.	Photosynthetic Capacity** Range	Photosynthetic Capacity** Ave.
Myers'	6.5	23	0.069-0.120	0.090	0.087-0.182	0.136
Allen's	7	32	0.065-0.215	0.111	0.030-0.260	0.131
Gorham's	9	34	0.044-0.230	0.144	0.031-0.222	0.131
Dil. Water	7	14	0.118-0.307	0.195	0.014-0.039	0.027

* $\triangle$DO/mg S.S./5 days

** $\triangle$ DO/mg S.S./2 hrs., 350 ft candles light

3. Effect of the pH of Gorham's Medium Upon Algal Activity

Because the data of the previous section had pointed out that there might be a relation between the pH of the culture medium and the activity of the algae, further studies were carried out using Gorham's medium.

It had been found that the range in pH values for cultures in Gorham's medium was 8.7 to 9.1 when these cultures were aerated with air, and pH 7.0 to 7.8 when aerated with the 1.25% CO_2-air mixture. Therefore, aside from changes in the soluble mineral composition because of changes in pH, this medium could be used to study the effect of near neutral and alkaline pH values on the activity of Chlorella.

A series of 34 tests were made with algae from the alkaline Gorham's medium (aerated with air), 15 tests using cultures 3-8 days old and 19 tests with cultures 19-23 days old. A second series of 34 tests were made with the neutral Gorham's medium (aerated with a 1.25% CO_2-air mixture), 18 tests using cultures 3-7 days old and 16 tests using cultures 16-23 days old. The average values obtained for each media and range in ages are summarized in Figure II-2 as the amount of oxygen used per mg of suspended solids (Δ DO/mg S.S.).

The data indicate that there was a loss of 24% in the activity of algae from older cultures at pH 8.7-9.1 as compared to younger cultures (0.124 vs. 0.163 Δ DO/mg S.S.), whereas algae from cultures at pH 7.0-7.8 had a difference of only 11% between old and young cultures (0.151 vs. 0.170 Δ DO/mg S.S.). When expressed as a comparison of cultures of similar ages, at different pH values, there was only a 4% difference between young cultures at pH 7.0-7.8 and pH 8.7-9.1, whereas, algae from old cultures of pH 7.0-7.8 were 18% more active than algae from similar cultures of pH 8.7-9.1.

Thus, it is indicated that the pH of the culture medium will affect the activity of the algae, the effect being more pronounced as the age of the culture increases. However, this effect may not be the result of the difference in pH but may have been caused by other factors accompanying these pH changes, such as a lack of dissolved CO_2 at pH 9, or a change in the nitrogen utilization at the different pH values.

4. Studies with Chlorella Grown in Secondary Sewage Effluent

Because the data obtained from studies with the above synthetic media indicated that differences in the activity of the algae could be caused by variations in the medium, studies were made on Chlorella grown in different variations of autoclaved secondary sewage effluent.

Autoclaved Madison, Wisconsin, secondary sewage effluent, referred to as sewage effluent or S.E., was used as the culture medium for Chlorella in the same manner as the synthetic media. The cultures were aerated with a 1.25% CO_2-air mixture at least until growth was well established, usually 2 days. The cultures continuously aerated with the 1.25% CO_2-air mixture maintained a pH of 7.3-7.8 (original pH of sewage effluent was 7.8, while the pH of autoclaved effluent was about 9). Cultures which were aerated with only air after the initial period of growth, had a range of pH from 8.8 to 9.4. At these higher pH values considerable salts could be precipitated (40 - 90 mg/l of suspended solids and about 75% of soluble PO_4-P) (1), also, there was little or no dissolved CO_2 present. The amount of suspended solids formed when sewage effluent was autoclaved and then aerated for six days with the 1.25% CO_2-air mixture was about 40 mg/l, whereas about 65 mg/l were formed when only air was used. Corrections for these amounts of inorganic suspended solids were made in calculating the suspended solids of the algal cultures.

In general, the amount of algae obtained per unit of time from cultures aerated with the 1.25% CO_2-air mixture was about twice that from cultures aerated with air only. It has been previously shown in this and other laboratories that the growth of Chlorella in sewage effluent cultures could be almost doubled by the addition of 400 mg carbon, as CO_2, per day (88 cc CO_2/1/hr.) to the basic aeration of 1500 cc air/min./tube volume of 350 mg (1, 2). It was also shown that the growth of Chlorella in sewage effluent was not decreased by the loss from solution, as suspended solids, of salts precipitated by raising the pH to 9.3 (including the loss of 75% of the PO_4 - P) (1). It would seem, therefore, that the difference in the growth rates, or amount of algae obtained per unit of time from cultures aerated with air or 1.25% CO_2-air mixture, was due to the addition of CO_2 to the medium and not to maintaining any particular salts in solution at the lower pH values.

The tests run with the algae grown in sewage effluent were designed to indicate whether the activity of algae in the BOD test was different when cultured under conditions of CO_2-limited growth (pH 9) as compared to sufficient CO_2 (pH 7.5). Twenty samples run with algae from cultures 8 and 9 days old, in which the culture medium either still had more than 1 mg/l of NH_3-N available, or had been only 24 hours without this level of nitrogen in the medium, indicated that the ratio of activities was 1.15 for $\triangle$ DO/mg S.S. and 0.83 for $\triangle$ DO/10^9 cells (for comparisons made of activity expressed as ratio, at pH 7.5 to pH 9.0).

In order to test whether nitrogen starvation would affect the relative activity of algae grown at pH 9 or 7.5, BOD tests were made on 24 samples of Chlorella from sewage effluent cultures 12 and 13 days old. These algae had been cultured for several days after the NH_3-N content of the medium was less than 1 mg/l. The ratio, pH 7.5/pH 9, of the average activity factors was 1.12 for $\triangle$ DO/mg S.S. and 1.15 for $\triangle$ DO/10^9 cells.

To further test the possible effect of nitrogen on the relative activity of algae grown at pH 7.5 and 9, Chlorella was cultured in sewage effluent to which NH_4HCO_3 had been added to bring the concentration of NH_3-N up to 40-41 mg N/l. Algae from young (7 days old) pH 7.5 cultures were less active than from pH 9 cultures, the ratio being 0.89 for $\triangle$ DO/mg S.S. and 0.90 for $\triangle$ DO/10^9 cells. However, cells from older (17 days old) pH 7.5 cultures were more active, the ratios of average activities being 1.26 for $\triangle$ DO/mg S.S. and 1.09 for $\triangle$DO/10^9 cells.

Since one of the differences between pH 7.5 and pH 9 sewage effluent cultures was the difference in inorganic ions because more salts were precipitated from solution at the higher pH and these salts probably were less available to the algae as such, tests were made on sewage effluent which had these variable suspended solids removed. A sample of sewage effluent was raised to pH 11 with NaOH and the resulting floc allowed to settle over night. The supernatant medium was decanted and its pH adjusted to 7.4 with HCl. There was no additional precipitate formed in this medium when the pH of a sample was again raised to 11. After autoclaving, the NH_3-N content of this medium was 19 mg/l and, as such, was similar to autoclaved sewage effluent, except as to the content of soluble salts and absence of an inorganic precipitate. Chlorella was cultured in this medium with and without added CO_2. The results of 11 samples tested from cultures of pH 7.4 and pH 9.0 showed that the ratio of the average activity factors were 1.07 for $\triangle$ DO/mg S.S. and 0.86 for $\triangle$ DO/10^9 cells.

The average activity factor, $\triangle$ DO/mg S.S., for all sewage effluent cultures with added CO_2 was 0.294 (41 samples) whereas it was 0.266 (41 samples) for algae grown under CO_2 deficiency and a higher pH (ratio of 1.10). The average factor $\triangle$ DO/10^9 cells, was 1.46 for pH 7.5 cultures and 1.56 for pH 9 cultures (ratio of 0.93).

These results with algae cultured in modifications of sewage effluent have indicated, therefore, that, as in the case of the synthetic media tested, algae grown under conditions of sufficient CO_2 (pH 7-7.5) were more active per mg of suspended solids in the BOD test than algae from CO_2-dificient cultures (pH 9). This difference is apparently enhanced by increased age of the cultures, but does not appear to be related to the supply of nitrogen as NH_3-N, or to the presence or absence of salts which might be removed from solution at the higher pH value.

5. Comparative Studies with Chlorella and Microcystis.

a) Growth Rate Studies

Studies have been carried out comparing the rate of growth of Chlorella pyrenoidosa (Wis. 2005) and the blue-green bloom producing

alga, Microcystis aeruginosa (Wis. 1036) when these two algae are cultured in Gorham's medium.

The rate of growth of the algae was followed by counts of the number of cells/ml and calculated as follows:

$$K = \frac{\text{Log cell number at } t_2}{\text{Log cell number at } t_1} \cdot (t_2 - t_1)\text{(in days)}$$

Two different periods of time were used to compare the rate of growth: 3 to 5 days after inoculation (range of cell counts approximately 3,000,000 cells/ml to 10,000,000 cells/ml) and 5 to 12 days after inoculation (range of cell counts approximately 10,000,000 cells/ml to 28,000,000 cells/ml). It was found in preliminary experiments that these two periods generally had different rates of growth and could be used to compare the algae under different physiological conditions.

In two experiments with aerated Gorham's medium (pH 9) the growth rates for Chlorella were 0.22 and 0.25 for the younger cultures and 0.10 and 0.09 for the older cultures. In the same experiments, the growth rates for Microcystis were 0.22 and 0.19 for the younger cultures and 0.09 for the older cultures. When Gorham's medium was gassed with 1.25% CO_2 in air (pH 7), the rates of growth of Chlorella and Microcystis in young cultures were 0.33 and 0.24, respectively, while in cultures 5 to 7 days old the rates of growth were 0.15, and 0.20, respectively

Measurements of the suspended solids in the cultures during the period of 3 to 5 days growth from the time of inoculation showed that the suspended solids increased from approximately 40 to 110 mg/l. During the period 5 to 7 days in the pH 7 medium the suspended solids increased from 110 to 240 mg/l, whereas in the pH 9 medium the suspended solids increase for the 5 to 12 days period was only 110 to 300 mg/l.

b) Effects of pH and Age

A series of tests were carried out in which cultures of Chlorella and Microcystis of different ages from Gorham's medium were used in the BOD test (at least 16 samples per condition). The cultures were either aerated and were alkaline (pH range: 8.9-10.6) or were gassed with 1.25% CO_2 in air and were neutral (pH range: 6.5 to 7.8).

A summary of the data from BOD tests and tests of the photosynthetic capacity after 5 days' incubation in the dark is presented in Table II-2.

It can be seen from these data that, as has been mentioned previously, the activity of Chlorella decreases with the age of the cultures and this decrease in activity with age is more pronounced with algae from the pH 9 medium as compared to algae from the pH 7 medium. In contrast, it was found with Microcystis that, whereas the activity of the algae in the BOD test and the photosynthetic capacity of the algae decreased with the age of the cultures, the photosynthetic capacity of Microcystis from the more alkaline, pH 9, medium was much greater than that from the neutral medium.

c) Effect of Mixing Chlorella and Microcystis

Since some workers have been able to show antagonism between various species of algae (3, 4), a series of 16 tests were carried out in which different relative concentrations of Chlorella from Allen's medium and Microcystis from Gorham's (pH 9) medium were used separately and in combinations in the BOD and photosynthetic capacity tests.

From the activity of the algae when measured separately in these tests, calculations were made of expected activity rates of the various mixtures, depending upon the relative concentration of each alga. The results of 16 BOD tests of mixtures of these algae indicated an average $\triangle$ DO/mg S.S. of 0.102, the range being 0.069 to 0.133, or well within the range of activity measured for the algae separately. The results of the photosynthetic capacity tests, after 5 days' incubation in the dark, separately and in mixtures indicated that the measured rates for the mixtures were 99 to 108% of theoretical rates for such mixtures calculated from rates measured when algae were tested separately. Thus, one can say that the activities of Chlorella and Microcystis, when mixed in these tests, were additive and not antagonistic.

d) Long Term BOD Studies

The amount of oxygen used (BOD) and photosynthetic capacity of either Chlorella or Microcystis in seeded BOD dilution water have been followed at 1 to 3 day intervals for as long as 28 days. Four tests were run with Chlorella from 3 different media and 3 tests with Microcystis from Gorham's (pH 9) medium. In all tests with either alga, there was a gradual decrease in DO in the BOD bottles until zero DO was reached (7 - 12 days incubation). The photosynthetic capacity of the algae was measured up to and after the time the bottles became anaerobic.

Table II-2

Summary of the Effect of Age and pH on the BOD and Photosynthetic Capacity of Chlorella and Microcystis Grown in Gorham's Medium

(Averages of at Least 16 Tests per Condition)

		Chlorella		Microcystis	
Medium	Ages (Days)	BOD*	Photosynthetic Capacity**	BOD*	Photosynthetic Capacity**
Gorham's pH 7	3-8	0.170	0.152	0.165	0.085
Gorham's pH 7	19-24	0.151	0.111	0.075	0.073
Gorham's pH 9	3-8	0.163	0.093	0.129	0.164
Gorham's pH 9	19-24	0.124	0.042	0.083	0.109

*Δ DO/mg S.S./5 days

**Δ DO/mg S.S./2 hrs., 350 ft candles light

It was found that in the samples with Chlorella, the photosynthetic capacity of the algae gradually decreased after the bottles became anaerobic, but was still measureable for periods as long as 8 days under anaerobic conditions: Photosynthetic capacity of Chlorella decreased from 0.178 to 0.078 △ DO/mg S.S. during 8 days under anaerobic conditions in a typical experiment. In the tests with Microcystis, the photosynthetic capacity of the alga was maintained at a higher rate for a longer period of time: photosynthetic capacity of Microcystis decreased from 0.125 to 0.079 △ DO/mg S.S. during 19 days under anaerobic conditions in one experiment.

6. BOD of Other Algae

The effects of 3 other algal species on the BOD test have been studied. The first, Scenedesmus dimorpha, was isolated from a sewage oxidation pond at Lancaster, California, by Dr. R. G. Zehnpfennig and was chosen for study as a representative species of the types of green algae found in such installations.

It was found that this alga, Scenedesmus, could be readily cultured in Allen's medium for the BOD tests. The average effect on the BOD test in seeded dilution water of 37 tests indicated it had an activity factor of 0.129 △ DO/mg S.S. and an average photosynthetic capacity after 5 days in the dark of 0.131 △ DO/mg S.S.

A few tests (a total of 8) were run with the blue-green algae, Nostoc muscorum (Wis. 1013). This alga is well-known for the many studies of its ability to fix atmospheric nitrogen (5, 6). The average activity factor in the BOD test of this alga was 0.093 △ DO/mg S.S. and the average photosynthetic capacity after 5 days in the dark was 0.081 △ DO/mg S.S.

The alga, Gloeotrichia echinulata (Wis. 1052), was chosen as a representative of the blue-green algae that cause obnoxious blooms in lakes and are also nitrogen fixers. This alga was cultured in 3 different media: Gorham's, Gorham's minus the nitrogen source, and Allen's. The average activity factors in the BOD test were: Gorham's medium (16 tests), 0.091 △ DO/mg S.S.; Gorham's minus nitrogen (6 tests) 0.132 △ DO/mg S.S.; and Allen's (6 tests), 0.048 △ DO/mg S.S. The photosynthetic capacity of these algae after 5 days in the dark generally was found to be small for the majority of the samples tested.

As a result of this observation of the low photosynthetic capacity of Gloeotrichia, a series of daily BOD and photosynthetic capacity tests were carried out. In the first two tests in seeded dilution water of Gloeotrichia cultured in Gorham's medium, it was noted that the algae ceased to evolve oxygen on the 2nd or 3rd day when exposed to light. Within 48 hours of the time when the

algae ceased to photosynthesize, there was a sudden increase in the rate at which oxygen was consumed as compared to the rate of the first 3 to 4 days and to the rate of oxygen consumed in Chlorella tests run at the same time.

A third series of tests with Gloeotrichia from Gorham's (pH 9) medium were carried out with seeded dilution water, seeded Gorham's medium, and raw lake water as the BOD test medium. The results of the tests in seeded dilution water and seeded Gorham's medium are plotted in Figure II-3. The results of the tests in raw lake water were similar to these results, except the rate of oxygen used was greater; BOD activity factors for dilution water, Gorham's medium, and lake water were 0.144, 0.149, and 0.590 Δ DO/mg S.S./5 days, respectively.

From the data of Figure II-3 it can be seen that this alga, Gloeotrichia, seems to die after 3 to 4 days under the conditions of the BOD test (the point at which the algae cease to photosynthesize). Subsequently, the organic matter of the algae apparently becomes available to the bacteria present and an increased BOD activity results (a calculation of the projected BOD of the algae in dilution water during the period, 5 to 10 days, would give a BOD rate of about 0.375 Δ DO/mg S.S.).

Summary and Conclusions

1. The activity of Chlorella in the BOD test was found to vary considerably, averages of activity ranging from 0.09 to 0.19 mg O_2 used per mg suspended solids per liter, depending upon the medium in which the algae had been cultured.

2. The average and range of values obtained in the BOD test with Chlorella from different culture media are presented in tabular form.

3. It was found that the activity of algae from Myers' and Gorham's media decreased with increasing age of the cultures.

4. Studies of the effect of pH of Gorham's medium upon the activity of Chlorella in the BOD test indicated that algae from the more alkaline (pH 9) medium exhibited a greater decrease in activity with increasing age of the culture than algae from neutral (pH 7.0-7.8) medium.

5. Chlorella cultured in autoclaved secondary sewage effluent exhibited a higher activity in the BOD test (an average of 0.27 mg O_2 used per mg suspended solids) than algae from the synthetic culture media. Also, algae from neutral pH cultures were more active than algae from CO_2-deficient cultures (pH 9).

6. When comparative tests with Chlorella and Microcystis were made it was found that the rate of growth of these algae in the same medium (Gorham's pH 9) was remarkably similar.

7. Both Chlorella and Microcystis exhibited a decrease in activity with increasing age of cultures. However, Microcystis from Gorham's pH 9 medium had a higher photosynthetic capacity after 5 days' incubation than the same alga from Gorham's pH 7 medium.

8. When Chlorella and Microcystis were mixed for the BOD test it was found that their separate activities were merely additive and not antagonistic to each other.

9. Microcystis was found to be able to photosynthesize after long periods (up to 19 days) of anaerobic conditions as well or better than Chlorella.

10. The effects of Scenedesmus dimorpha and Nostoc muscorum on the BOD test were similar to those Chlorella and Microcystis.

11. Tests with Cloeotrichia echinudata indicated that this alga appears to die under the conditionsoof the BOD test, thereby causing increased bacterial respiration.

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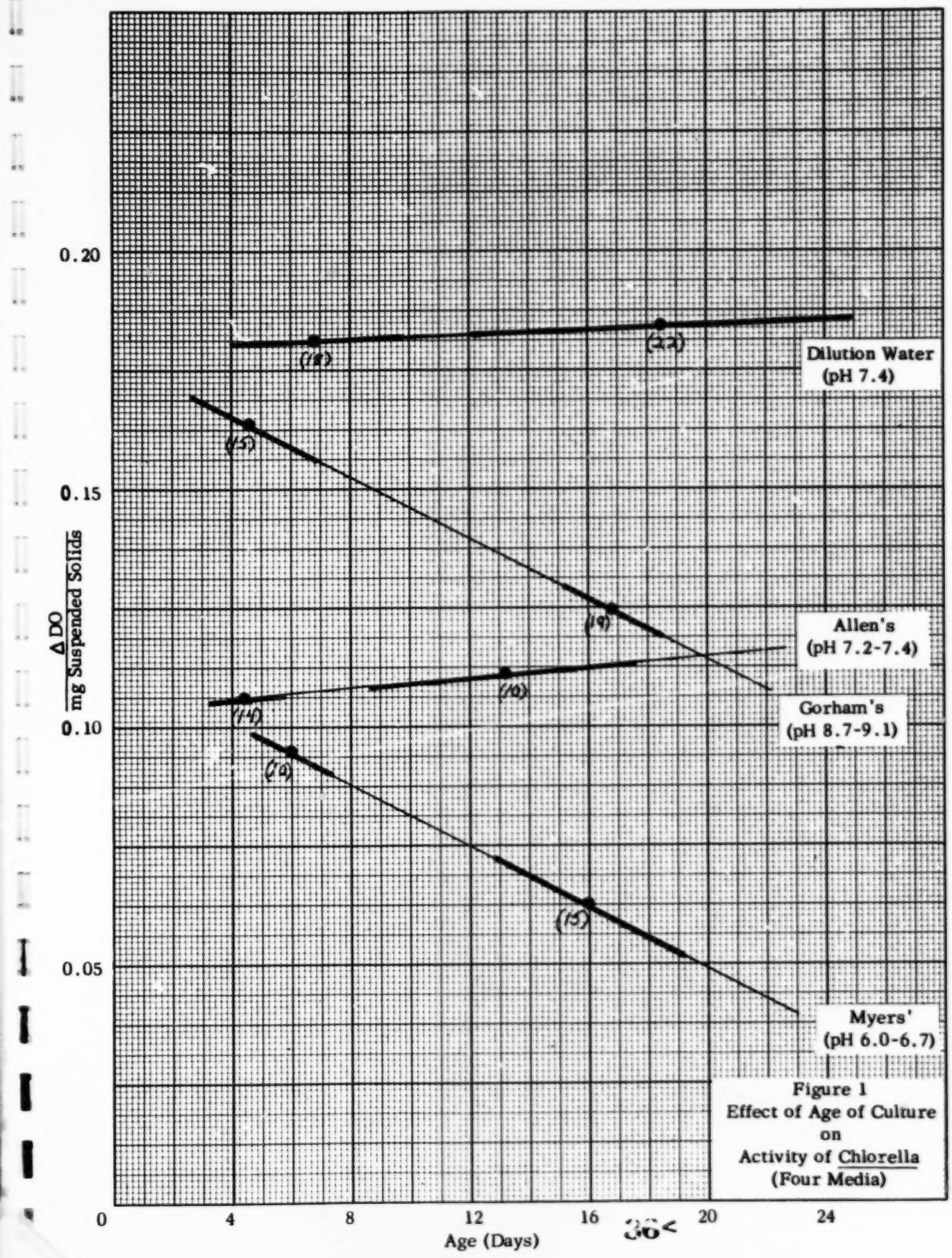

Figure 1
Effect of Age of Culture
on
Activity of Chlorella
(Four Media)

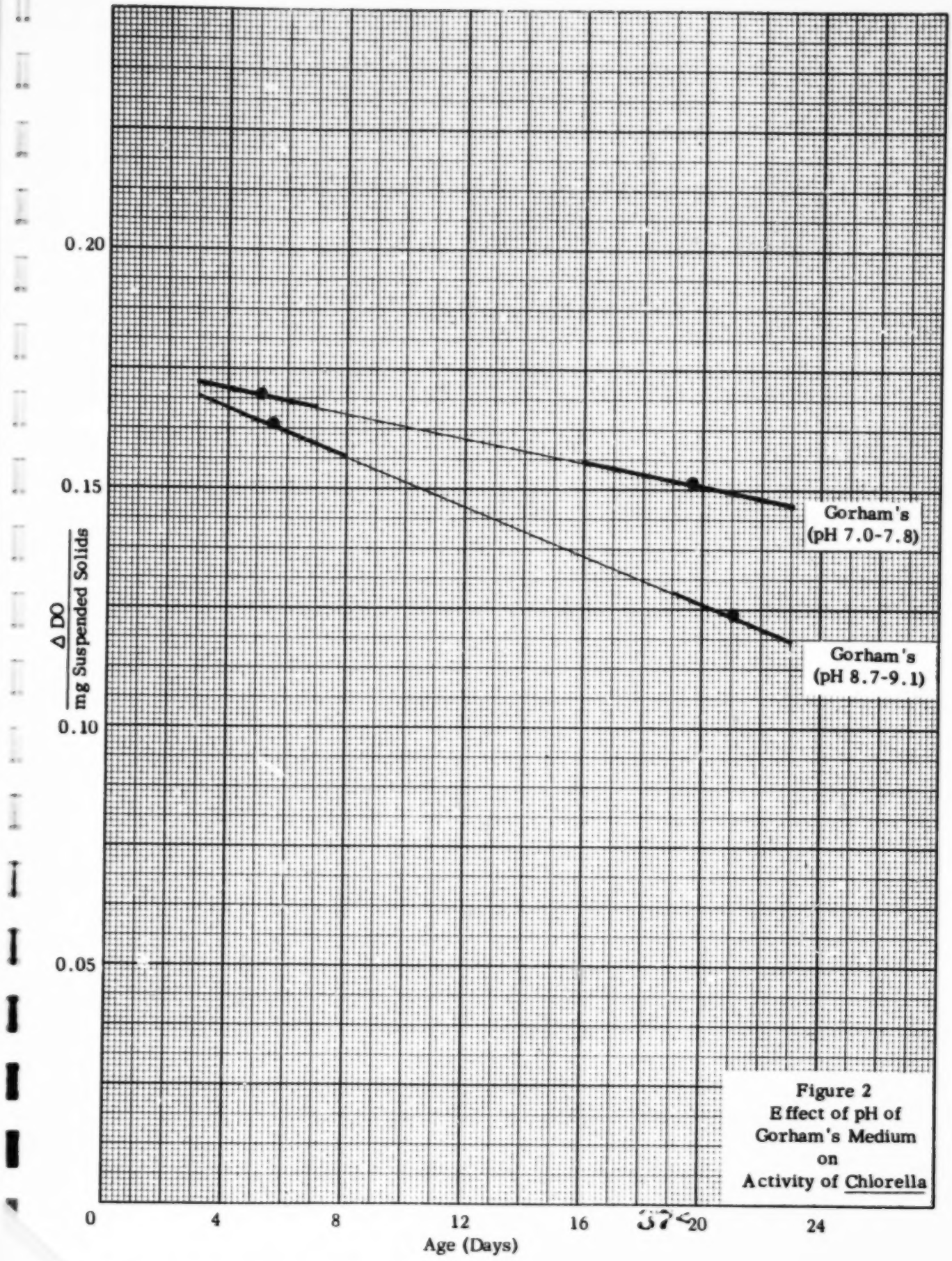

Figure 2
Effect of pH of Gorham's Medium on Activity of Chlorella

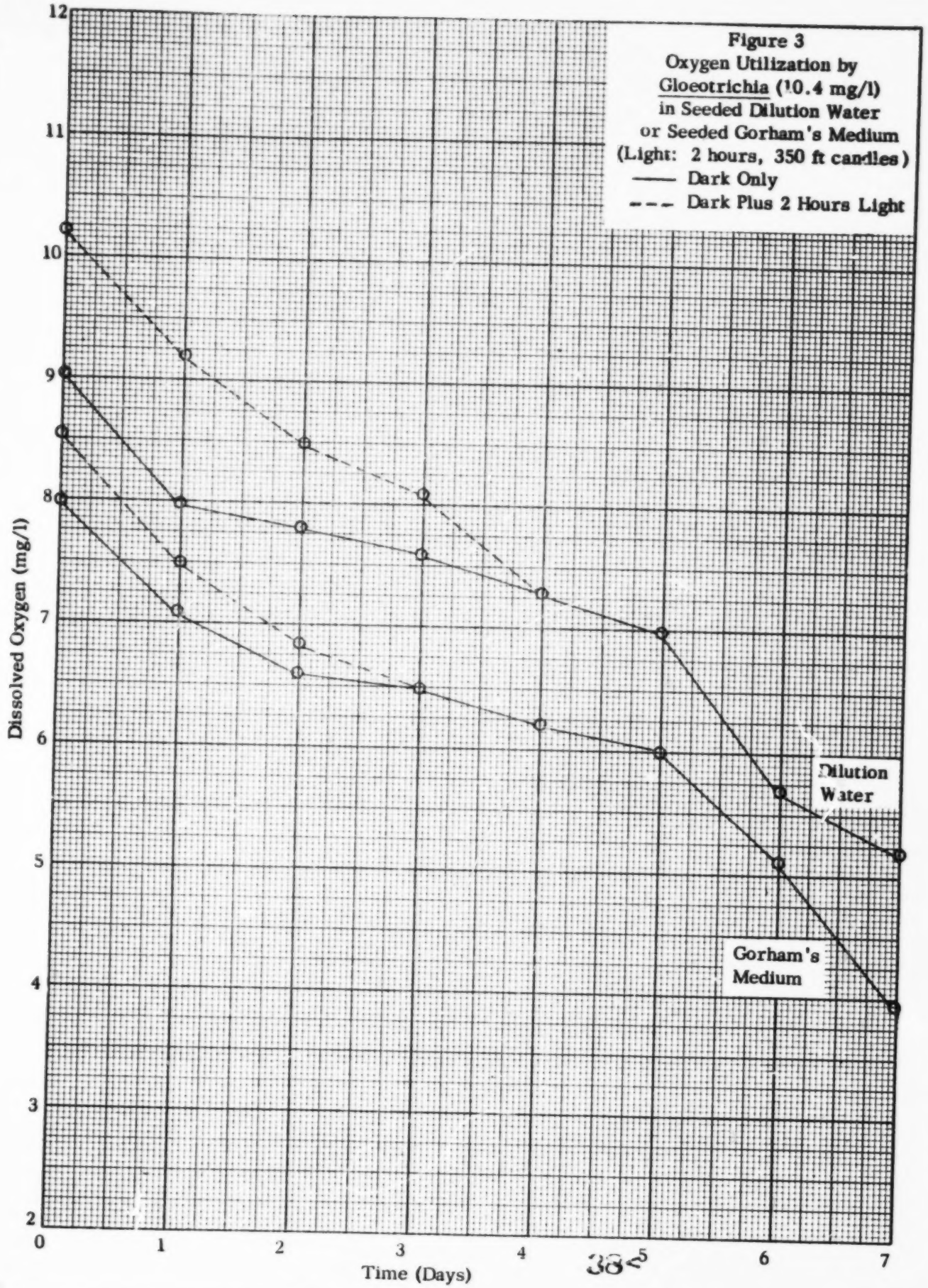
Figure 3
Oxygen Utilization by
Gloeotrichia (10.4 mg/l)
in Seeded Dilution Water
or Seeded Gorham's Medium
(Light: 2 hours, 350 ft candles)
—— Dark Only
- - - Dark Plus 2 Hours Light
Dissolved Oxygen (mg/l)
Time (Days)
Dilution Water
Gorham's Medium
12
11
10
9
8
7
6
5
4
3
2
0
1
2
3
4
5
6
7

THE EFFECT OF ALGAE ON BOD MEASUREMENTS

III. The Effect of Dilution Media on the BOD of Algae

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Introduction

The biochemical oxygen demand has been used since the end of the nineteenth century as a measure of the pollutional strength of sewages and industrial wastes which are discharged into natural waters in which aerobic conditions exist. The BOD test, which usually is defined as the amount of oxygen required by bacteria while stabilizing decomposable organic matter aerobically, takes into account the oxygen consumption of any living organisms present under conditions prescribed in the test procedure. Problems of pollution where algae are present are receiving increased attention, and because algae are living organisms which consume oxygen, their effect must be accounted for in the BOD test. The presence of algae in a water may affect the BOD value in three ways: 1) They represent the addition of organic matter capable of aerobic bacterial decomposition, thereby adding to the normal BOD, 2) by the respiratory activity of the live cells, thereby utilizing the dissolved oxygen of the dilution water and causing an added apparent BOD, and 3) by the photosynthesis of live cells in the light, thereby producing oxygen and lowering the expected BOD.

In attempts to make the BOD test most nearly approach the environmental conditions in natural waters, various dilution media were used by early investigators, culminating in the recommendation of the Standard Methods Committee of the Federation of Sewage Works Association (1) in 1940 to use as standard dilution water the mineralized phosphate buffered water of Theriault (2) supplemented with ammonia. Most investigators found that the use of natural waters as dilution media, in general, yielded higher BOD results than either distilled or buffered distilled waters, but results could not be standardized between one natural water and another.

Stream studies by Lueck, et al. (3) gave evidence that a large amount of algal cell material has an important effect on the capacity of rivers and streams to self-purify. Bartsch (4) states that although BOD reduction is usually accepted as a measure of treatment efficiency, it is almost meaningless when it involves algae-laden samples. He concluded that there is a pressing need to understand the real value of algae and to use them more effectively in waste treatment.

The purpose of the study reported here was to ascertain the effect of carrying out the BOD test in different media with algae present. The green alga Chlorella (Wisconsin strain) was used as the test organism because it is especially adaptable to various culture conditions; it can be compared with other algae with more restricted culture conditions; and it is an alga which is common in sewage treatment ponds, and, therefore, has importance in waste treatment and pollution problems. The effect of the dilution medium on the activity of Chlorella in the BOD test was measured by comparing standard dilution water, prepared according to the 1960 edition of Standard Methods, with various natural and synthetic waters.

Materials and Methods

The natural water used for comparison purposes was taken from Lake Mendota, Madison, Wisconsin, and stored in nineteen-liter carboys at 20° C for from one to fourteen days after being filtered through glass wool to remove filterable material.

The Chlorella pyrenoidosa (Wisconsin 2005) was cultured in Allen's media (5) with air containing two per cent carbon dioxide bubbling through and constant mixing with a magnetic bar. A volume of pure culture of about 30 ml was used to inoculate each experimental culture in a two-liter Erlenmeyer flask with 1500 ml of medium. The flasks were placed in a constant 23° C temperature culture room under continuous light of 150 foot candles. The concentration of the culture (mg/l) was normally measured by suspended solids using glass filter pad gooch crucibles and fifteen to thirty ml of algal suspension. The required volume of algal suspension to obtain a desired concentration of algae on resuspension was centrifuged; the supernatant was removed and the algae were resuspended in lake water or in a phosphate buffer solution. Seed used was collected as fresh sewage from the Madison Brittingham Park pumping station and placed at 20° C for from twenty to forty-eight hours.

Measured samples of algae were added to seeded or unseeded volumes of the various dilution waters in a two-liter graduate for mixing and filling of the BOD bottles. Triplicate bottles were always used for analysis. Six bottles for each algal concentration were incubated, and at least two different concentrations of algae were used in each experiment. At the end of the dark incubation period the bottles were removed from the 20° C room. Three bottles for each concentration of algae were placed in 350 ft. candles of light for two hours, after which time they and the remaining three bottles were analyzed for dissolved oxygen by the Alsterberg (azide) modification of the Winkler method. The oxygen used by the seeded dilution water and by the seeded dilution water plus algae was determined in this manner; the difference in oxygen depletion was referred to as being caused by the alga. The amount of oxygen

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produced by photosynthesis in the two-hour light period was obtained by using the difference between light and dark dissolved oxygen values. This photosynthetic capacity was used as an estimate of the physiological condition of the Chlorella after the incubation period.

Results

The dissolved oxygen uptake or production is reported as the increment of DO in mg/l per mg of suspended solids of algae present in the BOD bottle. This term, Δ DO per mg suspended solids, is termed the activity factor of the algae for samples incubated in the dark for five days; and is termed the photosynthetic capacity for dark plus two hours of light samples. Table III-1 summarizes the effects of various dilution media on the BOD of algae.

1. Comparison of Lake Water and Standard Dilution Water

Table III-1 shows that in 41 samples (a sample consisted of the average value for three BOD bottles at one algal concentration) using fresh lake water as dilution medium an average activity factor of 0.254 Δ DO was found, while in 45 samples of standard dilution water an average of 0.107 Δ DO was observed. The average activity factor of the algae in fresh lake water was, therefore, 2.28 times the activity factor of the algae in standard dilution water. The photosynthetic capacity of the Chlorella in lake water was in most instances less than that in standard dilution water, but it was sufficiently high to indicate that about as many algae were alive in the lake water as in the standard dilution water at the end of five days. The photosynthetic capacity was used merely as an indication of the viability of the algae at the various times analyzed because variables such as mixing and light to all parts of the bottle are such that a quantitative value of photosynthetic capacity could not be given.

Lake water was aged for one to two days for fresh lake water and from three to seven days for aged lake water. The BOD of the lake water itself with no seed or algae added was reduced in all cases after ageing and the average BOD of the algae was also reduced, from 0.254 Δ DO in fresh lake water to 0.187 Δ DO in aged lake water in comparison with 0.107 Δ DO in standard dilution water. Using unseeded lake water with algae added, there was a small difference in activity, an average of 0.220 Δ DO for 25 samples of unseeded fresh lake water compared to 0.254 for 41 samples of seeded fresh lake water. When aged lake water was used the activity factors in the seeded and unseeded water were respectively 0.187 Δ DO and 0.210 Δ DO.

Table III-2 shows the effect of the age of the algal cultures on the BOD of the algae in lake and standard dilution waters. The algae used was cultured twelve or more days (referred to in Table III-2 as Chlorella + 12 days) for the

Table III-1

SUMMARY OF THE EFFECT OF THE DILUTION MEDIUM ON THE BOD OF CHLORELLA

Chlorella (5-8 days old)

Note: All values of D.O. in this and subsequent tables are given in mg/l.

Dilution Medium	Number of Samples	D.O. per mg Suspended Solids in 5 Days Dark		+ 2 Hours Light	
		Average	Range	Average	Range
Fresh Lake	41	0.254	0.167-0.417	0.111	0.059-0.162
Fresh Lake (no seed)	25	0.220	0.164-0.324	0.110	0.044-0.174
Aged Lake	34	0.187	0.112-0.388	0.106	0.036-0.157
Aged Lake (no seed)	6	0.210	0.146-0.345	0.113	0.062-0.198
Standard Dilution	45	0.107	0.063-0.166	0.127	0.055-0.194
2/3 Conc. Standard Dilution	8	0.102	0.062-0.121	0.158	0.144-0.181
Lake to Standard Dilution 1:1	8	0.190	0.144-0.239	0.152	0.059-0.218
Lake to Distilled 1:1	6	0.188	0.169-0.220	0.121	0.066-0.142
Deionized Lake	8	0.117	0.050-0.200	0.187	0.117-0.230

Table III-1 (cont'd)

SUMMARY OF THE EFFECT OF THE DILUTION MEDIUM
ON THE BOD OF CHLORELLA

Chlorella (5-8 days old)

Note: All values of D.O. in this and subsequent tables are given in mg/l.

Dilution Medium	Number of Samples	△ D.O. per mg Suspended Solids in 5 Days Dark		+ 2 Hours Light	
		Average	Range	Average	Range
Autoclaved Lake + CO_2	10	0.205	0.174-0.252	0.134	0.081-0.183
Autoclaved Lake	8	0.197	0.097-0.300	0.116	0.107-0.157
Lea-Nichols	8	0.096	0.057-0.144	0.146	0.103-0.167
Allen's	3	0.115	0.112-0.117	0.130	0.125-0.135
Standard Dilution + Lea-Nichols HCO_3	3	0.092	0.085-0.099	0.096	0.091-0.107
Standard Dilution + 4x Fe, PO_4, $MgSO_4$	3	0.070	0.063-0.079	0.080	0.070-0.091
Standard Dilution + A-Z + 30mg/l NO_3 - N	3	0.075	0.073-0.080	0.086	0.080-0.090
Standard Dilution + 30 mg/l NO_3-N	3	0.082	0.075-0.082	0.095	0.083-0.112
Gorham's ($FeCl_3$)	3	0.142	0.135-0.153	0.007	0.003-0.013

older algae as compared to 5-8 day old cultures for the younger algae. When the values in Table III-2 are compared, it is seen that the age of the algae had an effect on the BOD; the oxygen uptake per mg algae decreased with age. The activity of the older algae in lake water was higher (2.0 times) than in the standard dilution water. This parallels the observations made in these two media using the younger algae where the activity in lake water was 2.3 times that in standard dilution water.

Because Lake Mendota is a eutrophic, hard water lake and there was a possibility that such water alone would cause the increase in algal BOD, water was sampled from twelve southern and northern Wisconsin eutrophic, mesotrophic, oligotrophic, hard water vs. soft water lakes and the Wisconsin River. In all cases where a natural water was used, the $\triangle$ DO of the water due to the algae was higher than when standard dilution water was used.

The results of these tests confirmed earlier observations made by Lueck, et al. (3) and Fitzgerald (6) that the BOD of algae in a natural water was higher than in standard dilution water. Further tests were carried out, therefore, comparing lake water and standard dilution water with various artificial waters in order to determine why a natural water as a dilution medium would yield higher BOD results than a buffered distilled water.

2. Effect of Ionic Concentration

In order to determine the effect of ion concentration of the $\triangle$ DO values, a series of studies were made. When the total solids content of Lake Mendota water was determined to be an average of 200 mg/l total solids, and standard dilution water to be about 300 mg/l dissolved salts, an attempt was made to test a dilution medium of the same salt concentration as the lake water. Therefore a medium of two-thirds concentration of standard dilution water was used to bring the salt concentration to 200 mg/l. As noted in Table III-1, the average activity factor for eight samples of algae in this medium was 0.102 $\triangle$ DO in comparison to 0.107 $\triangle$ DO in standard dilution water and 0.254 $\triangle$ DO in fresh lake water.

Allen's medium, in which the algae used as inocula were grown, has a much higher total salt content than the standard dilution water as can be seen in Table II-3. In order to determine the effect of a major change in salt concentration or environment on the $\triangle$ DO values, Allen's medium was used as a dilution water. In this way the same level of salt concentration in both the culture and in the BOD bottle was maintained. As shown in Table III-1 for Allen's medium the average activity factor was 0.115 $\triangle$ DO, indicating that a change in the medium in which the algae were placed had little effect on the $\triangle$ DO value

Table III-2

SUMMARY OF THE EFFECT OF THE DILUTION MEDIUM ON THE BOD OF CHLORELLA

Dilution Medium	Number of Samples	Δ D.O. per mg Suspended Solids in 5 Days Dark		+ 2 Hours Light	
		Average	Range	Average	Range
		Chlorella (+ 12 Days)			
Fresh Lake	18	0.169	0.100-0.243	0.084	0.044-0.116
Standard Dilution	2	0.084	0.073-0.095	0.060	0.055-0.065
		Chlorella (5-8 Days)			
Fresh Lake	41	0.254	0.167-0.417	0.111	0.059-0.162
Standard Dilution	45	0.107	0.063-0.166	0.127	0.055-0.194

obtained. The activity factor of the algae in the medium in which they were cultured was about the same as that in the standard dilution water even though the latter had about one-sixth the ionic salt concentration of the Allen's medium. These results indicate that the total ionic concentration of the dilution medium has no immediate effect on the activity factor of the Chlorella.

Gorham's medium (7), another frequently used algal culture medium, was used as dilution medium in three samples. The composition of Gorham's medium is given in Table III-3. A high activity factor (0.142 △ DO) as shown in Table III-1 was obtained in this medium, but it was observed that the photosynthetic capacity was very low (0.007 average △ DO) when compared with any other medium tested. Because of the low photosynthetic capacity it would appear that the algae in this medium died in these few tests and, therefore, the BOD was raised by the utilization of oxygen in algal decomposition.

Lake water was diluted one to one with standard dilution water in eight tests and with distilled water in six tests. As seen in Table III-1, the average activity factors in both of these media were higher than in the standard dilution water, 0.190△ DO and 0.188 △ DO respectively. Again these results indicate that the total ionic concentrations of the waters have little effect on the BOD of the Chlorella because the lake to standard dilution water had a higher salt concentration than the lake to distilled, while the average△ DO value was nearly the same. Lake water diluted with various waters, with an without added salts, gave uniformly high values when compared to standard dilution water and dilutions of it.

3. Effect of Biological Environment of Lake Water

Tests were carried out to observe the effect of the biological environment on the △DO of Chlorella. Several investigators of the BOD test have suggested that the bacteria or certain growth promoting substances of stream water might cause natural waters to give a higher BOD value. Therefore, in efforts to observe whether the bacteria and/or protozoa of the lake water were the cause for the higher BOD results, lake water which had been autoclaved to sterile conditions (15 pounds pressure for 15 minutes) was used for dilution medium for a series of△ DO tests. In ten tests carbon dioxide was bubbled through the autoclaved water to dissolve the precipitate of carbonates and to lower the pH. In eight other tests no carbon dioxide was added and the water was filtered to remove the precipitate before the water was used for△ DO tests. As seen in Table III-1, in both series of tests the activity factor of the Chlorella was high when compared to standard dilution water. The photosynthetic capacity was in the same range as the standard dilution water, indicating comparable algal photosynthesis. This series of experiments indicates that the organisms of the lake water had only a slight effect on the activity of the Chlorella during the 5-day BOD test.

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Table III-3

COMPOSITION OF DILUTION WATERS IN MG/LITER

Salt	Standard Dilution Water	Lea-Nichols	Allen's	Gotham's
$(NH_4)_2SO_4$		2.50		
NH_4Cl	1.7		50	
$NaNO_3$			1000	496
KH_2PO_4	8.5			
$Ca_3(PO_4)_2$		0.01		
K_2HPO_4	21.75		250	39
$Na_2HPO_4 \cdot 7H_2O$	33.4			
KH_2PO_4		0.085		
Fe Citrate				6
$FeCl_3$	0.25		3	
$MgSO_4 \cdot 7H_2O$	22.5	0.01	513	75
$CaCl_2$	27.5		50	27
$Na_2SiO_3 \cdot 9H_2O$				58
Citric Acid				6
EDTA				1
Na_2CO_3				20
$NaHCO_3$		300		
Total Salts	297.5	302.605	1866	728

4. Effect of Supplemental Elements

A series of tests was run using standard dilution water supplemented with a variety of mineral elements and compounds in an attempt to see if the addition of minor elements, higher concentrations of major elements, or higher buffer capacity had an effect on increasing the BOD of the algae. In all tests, as noted in Table III-1, the activity factors ranged from an average of 0.070 to 0.092 $\triangle$ DO. No increase in activity therefore was obtained by the addition of the various substances such as Lea-Nichol's bicarbonate, $FeCl_3$, phosphate buffer solution (composed of the phosphates in the standard dilution water as in Table III-3), $MgSO_4$, A-Z minor element mixture (8), and nitrate-nitrogen.

5. Sliding Effect

The "sliding effect" (lower BOD values with decreasing dilutions), recognized as early as 1918 and identified by Lea (9) as attributable to low phosphate concentration in the dilution media used, was observed in most cases in which lake water and the standard dilution water was used. The Lea-Nichols dilution water suggested by Lea to eliminate the "sliding effect" was tested. Although a slight sliding effect was noticed in only one set of three in which the Lea-Nichol's water was used, the average activity factor (0.096 $\triangle$ DO as seen in Table III-1) was low even when compared to the standard dilution water.

6. Daily and Long Term BOD Tests

Several tests were made to establish the rate of oxygen uptake by the algae on a daily basis and for an extended period of time (up to 16 days). Dark-incubated samples and dark-incubated samples terminating with two hours of light were analyzed each day or every two days. Figures III-1 and III-3 show the oxygen utilization of Chlorella in seeded standard dilution water as compared to seeded lake water that had been filtered through glass wool. The rate of oxygen utilization by algae was quite uniform in both standard dilution water and lake water, although the rate in lake water increased more rapidly than in the standard dilution water. The effect of increasing the algal concentration is seen in Figures III-2 and III-3 when 15.1 and 30.1 mg/l algae were used respectively. After the second day the rate increased more rapidly with the higher concentration, but at the end of five days the activity factor of the higher concentration of algae was 0.200 $\triangle$ DO in comparison with 0.268 $\triangle$ DO for the lower, thus showing a large sliding effect. The utilization of oxygen by the seed was similar in the standard dilution and the lake waters.

Figure III-2 shows the photosyathetic capacity of the Chlorella after a specified number of days. This figure shows that the photosynthetic capacity of the algae in lake and standard dilution waters are approximately the same.

The increased rate of oxygen utilization in the dark in the lake water had little effect on the photosynthetic capacity of the algae. Because Fitzgerald (6) found that dead algal cells had a higher BOD than live cells, it would appear that a larger number of algae did not die in the lake water than in the standard dilution water because the photosynthetic capacity remains about the same. Therefore, the effect of the lake water on increasing the BOD of the algae would not seem to be caused by greater death of the algae in lake water.

One long term BOD experiment was run for 16 days to determine the rate of oxygen utilization during this period as compared to the usual 5-day period. Figure III-4 shows the utilization of oxygen by the algae after dark incubation and after dark incubation plus two hours of continuous light. The bottles containing algae, seed, and lake water became anaerobic after about nine days time. The algae present, however, continued to produce oxygen after exposure to light throughout the sixteen days of the experiment. The bottles with algae, seed, and standard dilution water did not become anaerobic until the sixteenth day, but the oxygen production by photosynthesis became sporadic after nine days. This indicates some loss in viability.

7. Effect of Deionized Water

The final series of tests were carried out using as a dilution medium lake water, which was run through anionic and cationic exchange resins. The lake water was sampled as usual from Lake Mendota, filtered through glass wool, and then passed through a cationic exchange column one inch in diameter and packed with 20 inches of Amberlite IR-120 strongly acidic resin and through an anionic exchange column with the same dimensions packed with 20 inches of Amberlite IRA-400, a strongly basic resin. Lake water passing through these columns was reduced in solids content from 200 mg/l to about 10 mg/l. This final effluent was then made up to standard dilution water by adding the usual salts. As seen in Table III-1, the $\triangle$DO of this water was very close to that of the standard dilution water made of normal distilled water. This indicates that something was removed from the lake water on passing through the columns that caused the high BOD of the lake water.

Summary and Conclusions

1. The activity of Chlorella, measured in units of $\triangle$DO per mg suspended solids of the algae, when lake water was used as a dilution medium was greater than that in standard dilution water.

2. The photosynthetic capacity of the algae after 5 days' incubation in the dark in lake water as a dilution medium was nearly the same as that in the standard dilution medium. It is concluded, therefore, that the algae did not die at a faster rate in the lake water than in the standard dilution water.

3. Ageing and sewage seeding of the lake water had little effect on the △ DO of the algae in that medium.

4. Older algae (as measured by the time in culture medium) gave a lower △ DO value than young algae in both lake and standard dilution water.

5. The addition of minor and major elements and added buffer capacity to the standard dilution water had no effect on the △ DO values obtained.

6. The organisms present in the natural water had little effect on the activity of the algae.

7. A dilution water identical to that of the medium in which the algae were grown showed no higher BOD values than the standard dilution water.

8. The total ionic concentration of the dilution medium had little effect on the activity of the algae in BOD test.

9. Deionized lake water made up to standard dilution water with the appropriate chemicals gave △ DO values comparable to the standard dilution water made up with distilled water. It is, therefore, concluded that some heat stable growth factor that cannot pass through the ion exchange column affected the activity of the algae or that the natural ratios of ions present is essential for maximum activity of the algae.

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FIGURE 1.

OXYGEN UTILIZATION OF CHLORELLA
DURING 5-DAY B.O.D. TEST

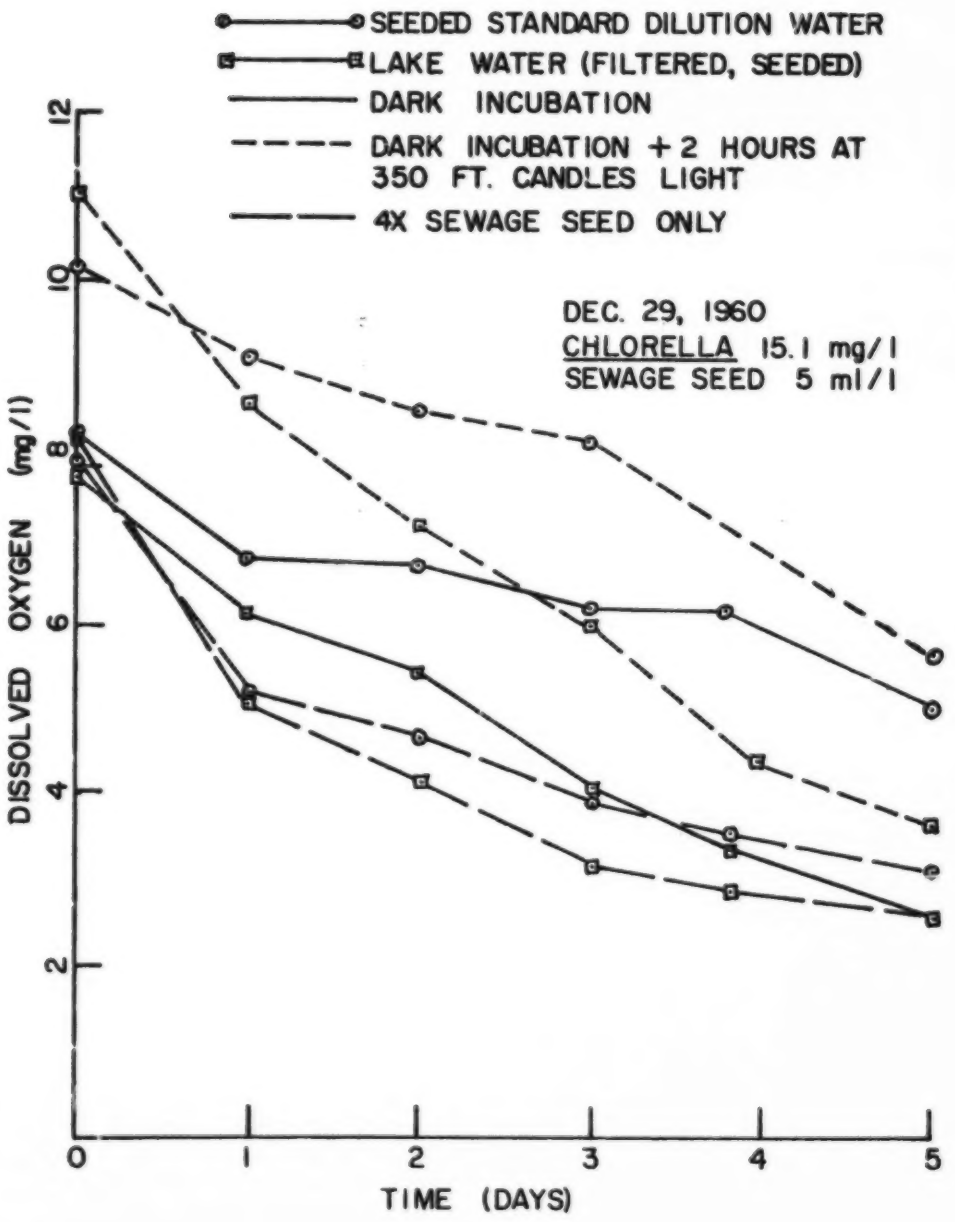

FIGURE 2.
PHOTOSYNTHETIC CAPACITY OF CHLORELLA
350 FT. CANDLES LIGHT FOR 2 HOURS

CHLORELLA 15.1 mg/l
SEWAGE SEED 5 ml/l

DEC. 29, 1960

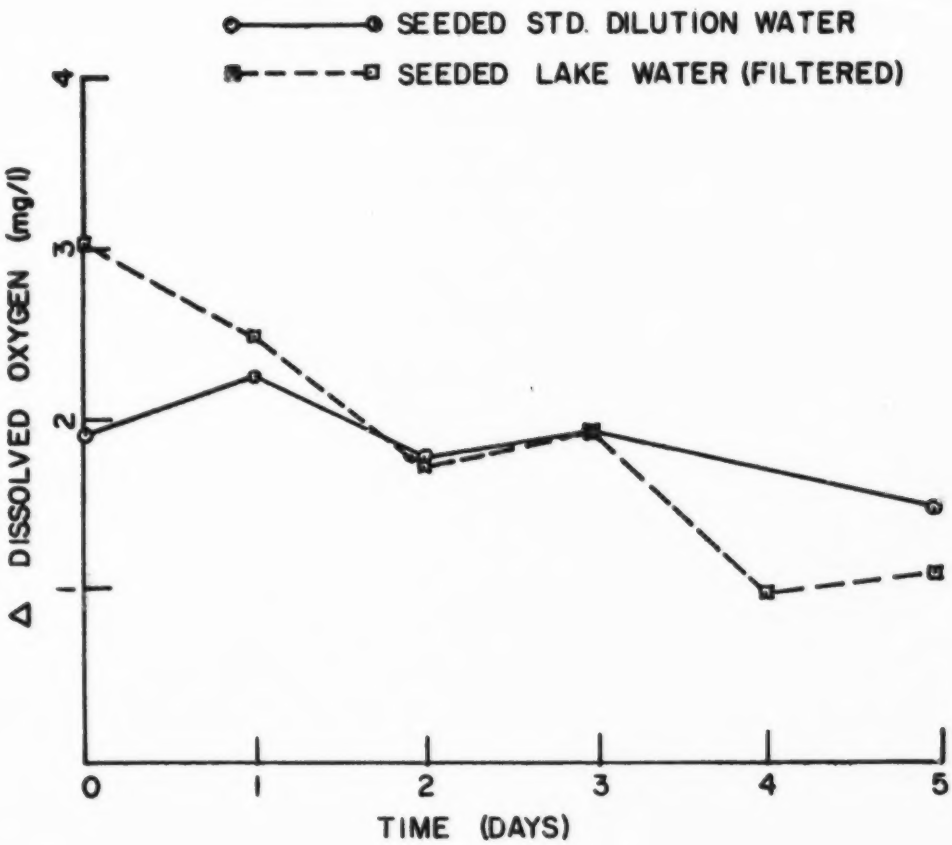

FIGURE 3

OXYGEN UTILIZATION OF CHLORELLA
DURING 5—DAY B.O.D. TEST

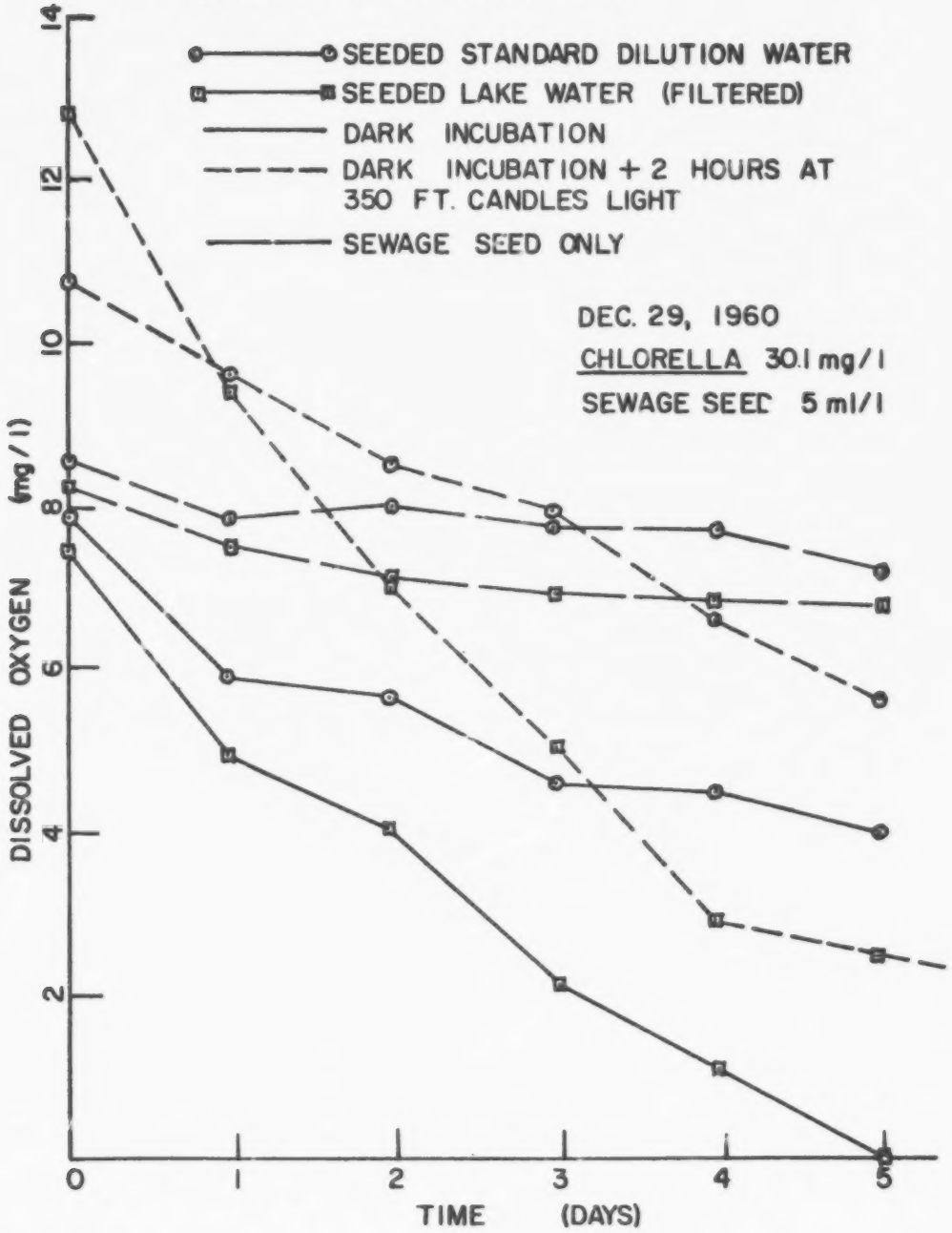

FIGURE 4

LONG TERM OXYGEN UTILIZATION OF CHLORELLA DURING 5—DAY B.O.D. TEST